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CELLULAR AND MOLECULAR IMMUNOLOGY

THIRD EDITION

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CHAPTER EIGHTEEN

IMMUNITY TO TUMORS

Malignant tumors, or cancers, grow in an uncontrolled manner, invade normal tissues, and often metastasize and grow at sites distant from the tissue of origin. In general, cancers are derived from only one or a few normal cells that have undergone malignant transformation. The abnormal growth behavior of malignant tumors is the reflection of complex abnormalities in physiology that result from expression of mutated or viral genes and/or deregulated expression of normal genes. In addition to the abnormal expression of molecules that contribute to malignant behavior, cancer cells frequently express mutated or dysregulated genes whose products do not contribute to growth or invasive properties of the tumor. The increased frequency of mutations in such cancer cells may be related to fact that the carcinogenic agents that promote the development of cancers, such as ionizing radiation and reactive chemicals, are not selective and may damage DNA anywhere in the genome. Cancers can arise from almost any tissue in the body. Those derived from epithelial cells, called carcinomas, are the most common kinds of cancers. Sarcomas are malignant tumors of mesenchymal tissues, arising from cells such as fibroblasts, muscle cells, and fat cells. Solid malignant tumors of lymphoid tissues are called lymphomas, and marrow and blood-borne malignant tumors of lymphocytes or other hematopoietic cells are called leukemias.

A variety of clinical and pathologic evidence indicates that tumors can stimulate immune responses. A common histologic observation that suggests that tumors may be immunogenic is the presence of mononuclear infiltrates, composed of T cells, natural killer (NK) cells, and macrophages, surrounding many tumors. Such infiltrates may develop at any site of tissue injury, but they are more frequently present around certain types of tumors, including testicular seminomas, thymomas, medullary breast carcinomas, and malignant melanomas in the skin, independent of the presence of other inflammatory stimuli, such as infection or tissue necrosis. Another histopathologic indication that tumors stimulate immune responses is the frequent finding of lymphocytic proliferation (hyperplasia) in lymph nodes draining sites of tumor growth. Furthermore, there is often evidence of cytokine effects in tumors, such as increased expression of class II major histocompatibility complex (MHC) molecules and intercellular adhesion molecule-1 (ICAM-1), suggesting an active immune response at the site of the tumor. The spontaneous regression of some tumors also suggests that host immune responses to tumor cells can occur, although there are many other explanations for this phenomenon.

The first clear example that tumors can induce protective immune responses came from a now classic set of studies performed in the 1950s with

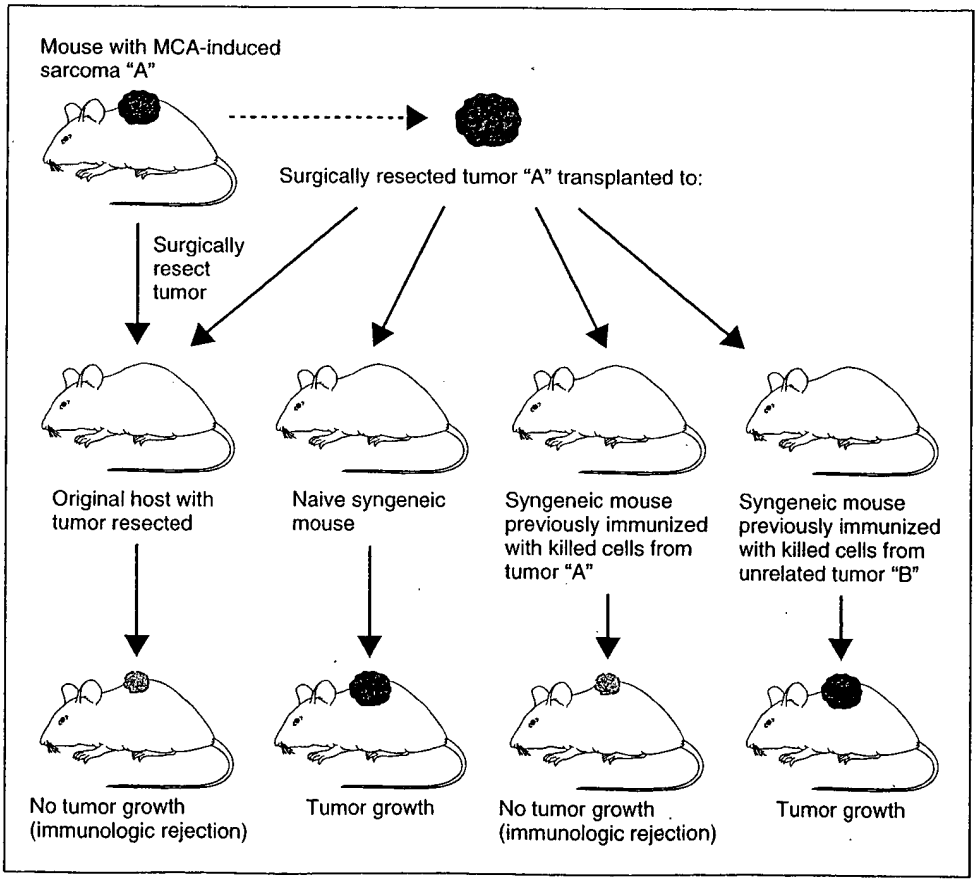


FIGURE 18-1. Experimental demonstration of tumor immunity: Specific immunologic rejection of chemically induced sarcomas. A mouse treated with the chemical carcinogen methylcholanthrene (MCA) develops a sarcoma. If this tumor is resected and transplanted into a normal syngeneic mouse, the tumor will grow. In contrast, the original tumor-bearing animal that was cured by surgical resection will reject a subsequent transplant of the same tumor. Injection of killed cells from the same tumor into a syngeneic mouse also induces protective immunity to that tumor, but injection of killed cells from an unrelated tumor does not.

rodent tumors induced by chemical carcinogens or radiation. In a typical study of this kind, a sarcoma is induced in an inbred mouse by painting its skin with the chemical carcinogen methylcholanthrene (MCA). These MCA-induced sarcomas can be excised from the original host mouse and introduced into other mice, or back into the original animal. Transplantation of these tumors into other syngeneic mice is usually successful, and the tumors grow and eventually kill the new host. In contrast, reintroduction of the tumor into the original host results in a specific immunologic rejection of the tumor. Alternatively, the cells of a tumor from one mouse can be killed by irradiation and used to immunize a second syngeneic mouse. Subsequent introduction of live cells from the original tumor into the immunized mouse will result in immunologic rejection of the tumor transplant, but a mouse immunized with killed cells from an unrelated tumor will not reject a transplant of the first tumor (Fig. 18–1). Furthermore, protective immunity in one mouse, established by growth of or immunization with a tumor, can be transferred to another tumor-free animal by $CD8^+$ cytolytic T lymphocytes (CTLs) (Fig. 18–2). These experiments demonstrated that the *rejection of the transplanted tumors displays cardinal features of specific immune responses, including dependence on lymphocytes, specificity, and memory.* Furthermore, the experiments indicate that tumor cells express antigens, called **tumor antigens,** which can be recognized by the host immune system.

If malignantly transformed cells express molecules that act as foreign antigens in the host, it is possible that a physiologic function of the immune system is to recognize and destroy these abnormal cells before they grow into tumors, or to kill tumors after they are formed. This theoretical role for the immune system is called **immunosurveillance.** The idea originated with Paul Ehrlich early in this century and was expanded in the 1950s and 1960s by Macfarlane Burnet and Lewis Thomas. If the concept of immunosurveillance is valid, then immune effector cells, such as B cells, helper T

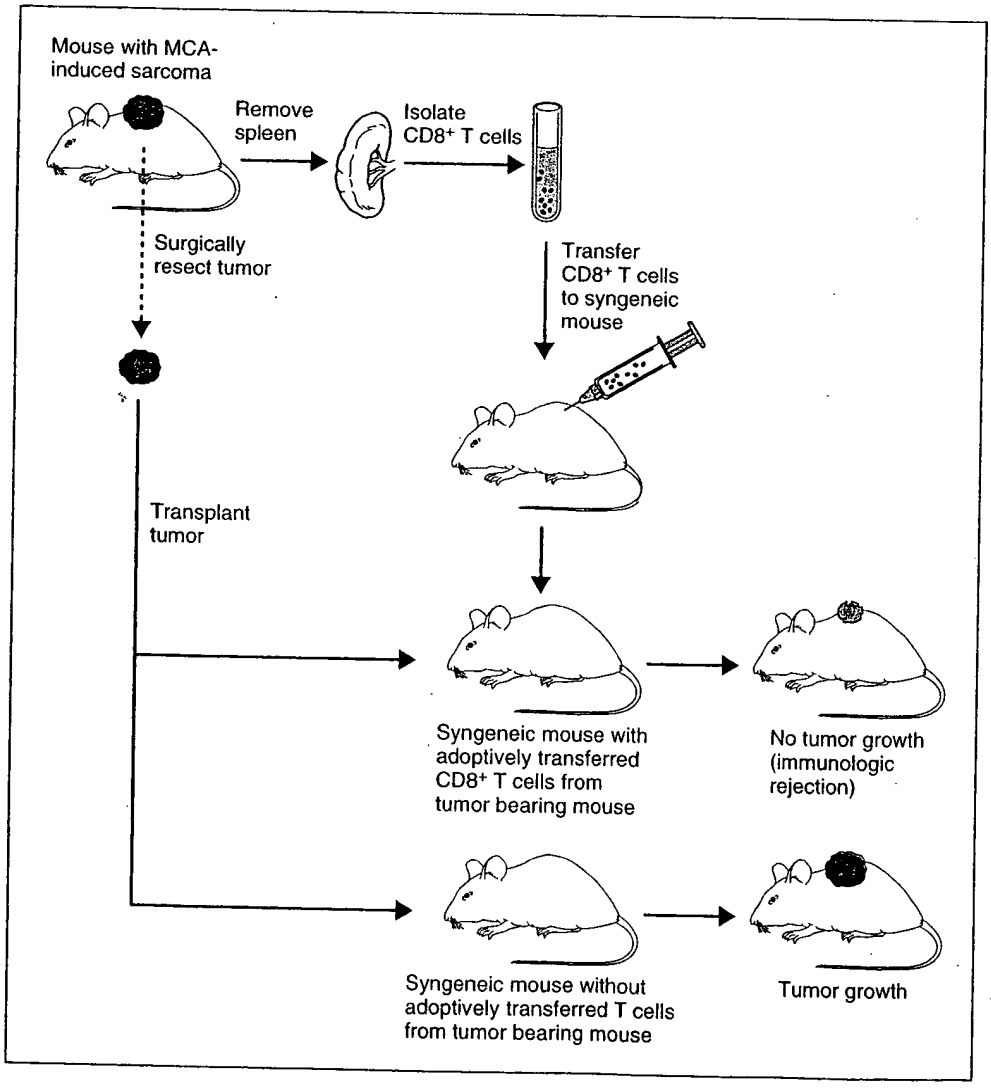


FIGURE 18–2. Immunity to transplanted tumors can be adoptively transferred by CTLs. Splenic $CD8^+$ T cells isolated from a mouse bearing a chemically induced sarcoma are transferred to a normal (tumor-free) mouse. This recipient mouse will subsequently reject tumor transplants from the original mouse, but tumor transplants will grow on another mouse which has not received any adoptively transferred CTLs. Transfer of $CD4^+$ T cells, B cells, or antibody does not transfer immunity.

cells, CTLs, and NK cells, must be able to recognize tumor antigens and mediate the killing of tumor cells. Although there still is little direct evidence that immunosurveillance protects individuals from many common tumors, certain observations support the validity of the concept. For example, immunodeficient individuals are more likely to develop certain types of tumors than normal individuals. Clinicopathologic correlations show that the presence of lymphocytic infiltrates in some tumors (e.g., medullary breast carcinomas and malignant melanomas) is associated with a better prognosis compared with histologically similar tumors without infiltrates. There is also abundant experimental evidence that tumors can stimulate specific T cell–mediated immune responses. Recently, several tumor antigens that are recognized by class I MHC–restricted CTLs *in vivo* have been identified as mutant or aberrantly expressed cellular proteins. These findings further support the idea that a function of CTLs is surveillance for and destruction of cells harboring mutated genes that could lead to, or be associated with, malignant transformation.

Immunosurveillance for tumors is often ineffective, as indicated by the fact that lethal cancers arise in immunocompetent individuals. It is therefore likely that immune responses to tumors are often weak, and the possible reasons for this are discussed later in this chapter. A major current focus of immunology and oncology research is the development of ways to augment host immune responses to tumors. This research is part of a broad field called **tumor immunology,** which encompasses the study of specific immune responses to tumors, the antigens on tumor cells that induce immune responses, immunologic effector mechanisms that kill tumor cells, and immunologic approaches to detecting, diagnosing, and treating cancers. The great progress we have made over the past 20 years in understanding the physiology of normal immune responses is already being applied to the important practical problems of prevention and treatment of tumors. In this chapter, we discuss these different aspects of tumor immunology, referring to the basic principles of recognition and effector phases of the immune response that we have already described in detail in previous chapters.

TUMOR ANTIGENS

Although tumors are derived from self tissues, they are more likely than normal cells to express molecules that are recognized as foreign by the immune system. As we have discussed, this is because of the high frequency of mutations in cancer cells, and dysregulated gene expression leading to the production of unmutated proteins not normally expressed. Furthermore, tumors caused by transforming (oncogenic) viruses may express viral proteins. The products of mutated, dysregulated, or viral genes may therefore be recognized as foreign by B and T lymphocytes because they were never expressed on self tissues prior to tumor development, or were expressed at sufficiently low levels that they did not induce self-tolerance. Such proteins can therefore stimulate specific immune responses to tumor cells. Alternatively, the products of mutated or dysregulated genes in cancer cells may contribute to the abnormal synthesis of nonprotein molecules, including carbohydrates and lipids, which can be recognized by B cells as tumor antigens. In addition, surface proteins peculiar to tumors may serve as targets for effectors of innate immunity, such as NK cells.

The fact that tumor cells express antigens that can stimulate immune responses in the host has been demonstrated in both experimental animal models and in human cancer patients. It has become apparent that patterns of expression of these antigens differ among different tumors and between tumors and normal tissues. Tumor antigens are often classified into different groups based largely on these patterns of expression:

1. **Tumor-specific antigens (TSAs)** are antigens expressed on tumor cells but not normal cells. These are the antigens most likely to evoke immune responses in the host because they are perceived as foreign. **Unique tumor antigens** are TSAs that are expressed on only one or a few clonal tumors, reflecting peculiar mutations that are characteristic of those tumors alone.
2. Some tumor antigens are also expressed concurrently on normal cells in the host, and expression may or may not be restricted to the type of tissue from which the tumor originated. These are called **tumor-associated antigens (TAAs).** Although some of these TAAs may induce immune responses in the host, they often do not, because of self-tolerance. TAAs are, in fact, usually defined by antibodies generated in animals of other species by immunization with tumor cells.

This portion of the chapter describes different types of tumor antigens and their roles in the biology of tumor-host interactions. We have categorized two main groups of tumor antigens based on the types of immunologic probes used to detect them, namely those recognized by T lymphocytes and those detected by antibodies. This categorization not only reflects methodologic approaches to the study of tumor antigens, but also, in some cases, indicates the types of immune responses which such antigens evoke in tumor hosts. Within each group there are examples of tumor-specific antigens, unique tumor antigens, and tumor-associated antigens. There is also some overlap in this classification, with some types of antigens capable of eliciting, and being detected by, both T cells and antibodies.

Tumor Antigens Recognized by T Lymphocytes

Tumor antigens that are recognized by T cells are the major targets of protective anti-tumor immunity in experimental animals, and probably in humans. The identification of these antigens has been a major focus of recent tumor immunology research, and it has relied on transplantation models in animals, the development of both mouse and human cloned CTL lines as probes for the antigens, cloning of genes from tumor complementary DNA (cDNA) libraries, and elution and analysis of peptides from tumor MHC molecules. These experimental approaches are described in Box 18–1. In Chapter 6, we discussed how MHC molecules display peptides derived from cellular proteins for surveillance by T cells. In particular, class I MHC molecules display peptides derived from cytosolic proteins in all nucleated cells. This is also true of tumor cells. Therefore, if a tumor cell produces a protein that is either not expressed by normal cells or is a mutated version of a normal protein, peptides derived from this tumor protein may be displayed by class I MHC molecules on the tumor cell surface, and recognized as foreign by host $CD8^+$ T lymphocytes (Fig. 18–3). Thus, tumor cells serve as antigen-presenting cells (APCs), displaying their own antigens to T cells. These antigens could

BOX 18–1. Identification of Tumor Antigens Recognized by T Lymphocytes

Tumor antigens recognized by T cells have been identified by the combination of three experimental approaches: transplantation studies of tumors in rodents, generation of cloned tumor-specific CTL lines, and the identification of peptides recognized by tumor-specific CTLs or the identification of the genes encoding these peptides.

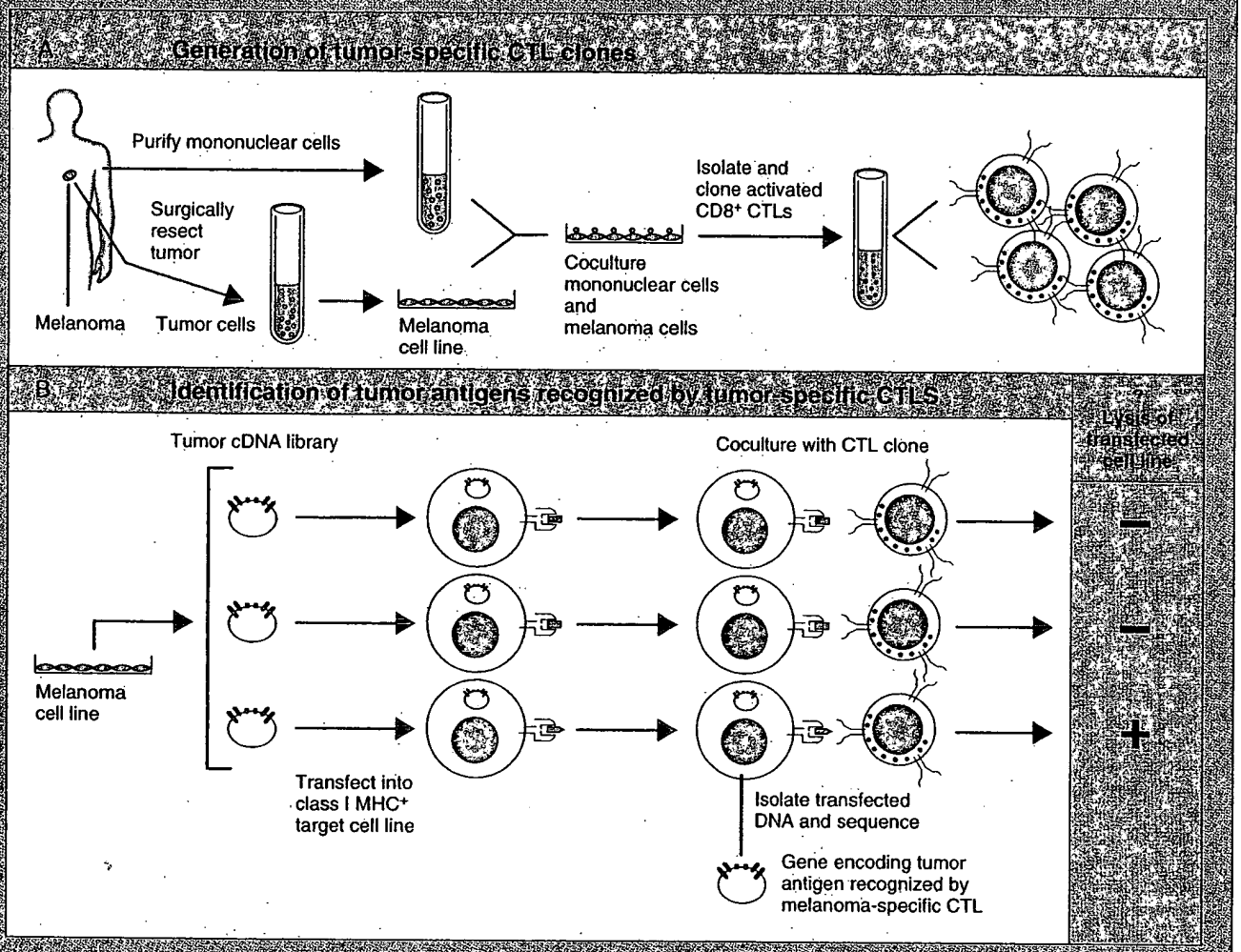
(1) *Transplantation studies of tumors in rodents were the first experiments to indicate the types of tumor antigens recognized by T cells.* Initial studies of this type used chemically induced rodent sarcomas (see Figs. 18–1 and 18–2) and they demonstrated the existence of tumor-specific transplantation antigens (TSTAs), which elicited highly specific immune responses. Another, more recent approach to characterize antigens that stimulate tumor rejection by CTLs relies on the *in vitro* mutagenesis of an established tumorigenic mouse cell line and isolation of non-tumorigenic mutants that are immunologically rejected when transplanted into syngeneic mice. In this system, the tumorigenic cell line does not express TSTAs and therefore grows unchecked when injected into a host animal, but the mutagenized cell line does express mutant protein antigens that induce its rejection. The role of CTLs in the rejection process in this model has been established by adoptive transfer experiments, and by the propagation of CTL clones that recognize the tumor, as discussed below. The actual genes encoding the rejection antigen in a few of these tumor variants have been cloned by methods described later, and they turn out to be cellular genes of unknown function with point mutations. This is a further demonstration that antigen-processing pathways in tumor cells can sample randomly mutated cytoplasmic proteins and thereby target the cells for lysis by CTLs.

(2) *The establishment of cloned CTL lines that recognize tumor antigens has been a key advance in the identification of tumor antigens.* This was first done in mouse models using the *in vitro* mutagenized tumor lines described earlier. Each CTL clone raised against one mutagenized tumor variant reacts against that variant but not against most others derived from the same parental tumor. This indicates that the antigens the CTLs recognize are highly diverse and unique to individual tumors, similar to the TSTAs described previously. More recently, many cloned CTL lines specific for human tumors, particularly melanomas, have been generated. Melanomas, which are malignant tumors of melanocytes, are often readily accessible surgically, resectable tumors, and are relatively easy to grow in tissue culture. CTLs specific for these tumors may be propagated, and subsequently cloned, by culturing T cells from a melanoma patient with lethally irradiated but antigenically intact cells derived from the patient's melanoma. The T cells can be isolated from peripheral blood, from lymph nodes draining the tumor, or from cells that have actually infiltrated the tumor *in vivo*. Since the T cells and the tumor are from the same individual, the MHC restriction of the T cells matches the MHC alleles expressed by the tumor. In these cocultures, CTLs that recognize peptide antigens displayed by the tumor cells are stimulated to grow, and single cell clones are propagated in interleukin-2 using limiting dilution techniques (see Figure).

(3) *The identification of the peptide antigens that induce CTL responses in tumor patients, and the identification of the genes encoding the proteins from which the peptides are derived, have relied on cloned tumor-specific CTL lines.* This has been accomplished in two ways. First, a direct biochemical approach has been used in which peptides bound to class I MHC molecules purified from melanoma cells are eluted by acid treatment and fractionated by reverse-phase high-performance liquid chromatography (RP-HPLC). The fractions are tested for their ability to sensitize MHC-matched non-tumor target cells for lysis by a tumor-specific CTL clone. This strategy relies on having a target cell that expresses the class I MHC molecules for which the CTL clone is specific but does not normally express the tumor antigen, and on the ability to load these cell surface MHC molecules with the exogenous HPLC-purified peptides. Peptide fractions that do sensitize the target cells are then analyzed by mass spectroscopy to determine their amino acid sequences. Once the peptide sequence is known, it may be possible to compare it with databases of protein sequences, to see whether it matches with any previously characterized protein and to determine whether there are point mutations from the normal sequences. Second, a genetic approach can be used to identify genes encoding tumor antigens (see Figure). This relies on preparing cDNA libraries from a tumor cell line that contains genes encoding all the tumor proteins. The library is prepared in molecular constructs that will allow constitutive expression of the genes when they are introduced into cell lines. Pools of DNA from such a library are transfected into a cell line expressing the appropriate class I MHC allele, and the transfected cells are tested for sensitivity to lysis by an anti-tumor CTL clone. The DNA pools that sensitize the target cell line presumably contain the gene that en-

Continued

codes the protein antigen recognized by the CTL clone. Multiple additional rounds of transfections using smaller and smaller subfractions of the DNA pool can lead to exact identification of the single relevant gene. The sequence of the gene can then be determined, and comparisons can be made with known genes. Synthetic peptides corresponding to different regions of the encoded protein can be tested for their ability to sensitize target cells in much the same way as is done in the peptide elution approach described previously. Both the biochemical and genetic approaches have been used successfully to identify human melanoma antigens that have stimulated CTL responses in the melanoma patients.



Clonal CTL lines specific for human tumors are used to identify specific tumor antigens. (A) $CD8^+$ T cells isolated from blood, lymph node, or tumors of melanoma patients are propagated in culture by stimulating them with melanoma cell lines derived from the patient's tumor. Single T cells from these cultures are expanded into clonal CTL lines. (B) DNA from melanoma gene libraries is transfected into class I MHC–expressing target cells. Genes that sensitize the target cells for lysis by the melanoma-specific CTL clones are analyzed to identify the melanoma protein antigens recognized by the patient's CTLs.

be encoded by the tumor cell genome or by viral genomes which are carried by the tumor cells. It is also possible that proteins released into the extracellular medium by viable or dying tumor cells, or whole tumor cells themselves, may be endocytosed into the class II MHC pathway of antigen processing by professional APCs, and class II MHC–associated peptides derived from these tumor cells can be displayed for recognition by $CD4^+$ helper T cells. It is not surprising that the tumor antigens that have been shown to be recognized by T lymphocytes include a wide variety of cellular and viral proteins.

The studies with chemically induced rodent sarcomas, such as those described in Figs. 18–1 and 18–2, established that tumors express transplantation antigens that can elicit specific immune responses. Although there was limited knowledge of antigen-presenting pathways at the time the first experiments of this sort were performed, we now understand that the tumor antigens in such studies are peptide–class I MHC complexes capable of stimulating CTLs. Experiments with multiple different rodent tumors, all induced by the same carcinogen, indicated that there was an enormous diversity of tumor transplantation antigens in that

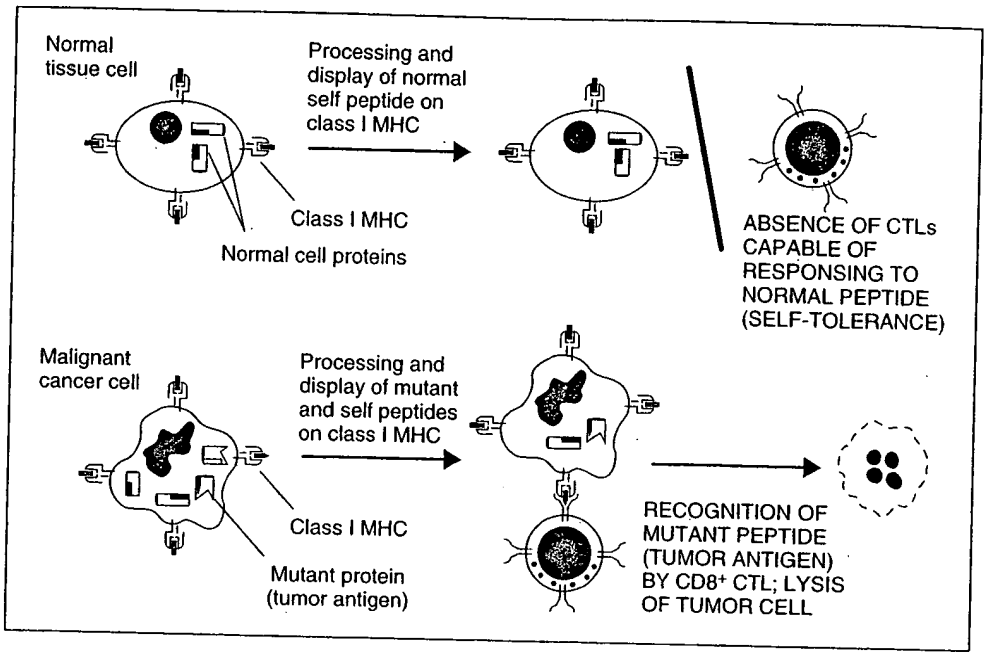


FIGURE 18–3. Immune surveillance for tumor antigens by class I MHC–restricted T cells. The class I MHC pathway of antigen presentation samples cytoplasmic proteins and displays peptides derived from those proteins on the cell surfaces. Because of self-tolerance, no T cells will respond to peptides from normal cellular proteins. CTLs will recognize peptides derived from mutant or aberrantly expressed proteins present in malignantly transformed cells, and the CTLs will kill the cancer cells expressing these proteins.

experimental tumor model, reflected by the specificity of the immune response to each individual tumor. For example, one MCA-induced sarcoma will not induce protective immunity against another MCA-induced sarcoma, even if both tumors are derived from the same mouse (Table 18–1). For this reason, such antigens are called **tumor-specific transplantation antigens (TSTAs).** In retrospect, this diversity is predictable since the carcinogens that induce these tumors may randomly mutagenize virtually any gene, and the class I MHC antigen-presenting pathway should be able to display peptides from each mutated protein in each tumor.

There are two important ways in which TSTAs on experimental animal tumors are relevant to the study of human cancers. First, the demonstration that tumors can be killed by tumor antigen-specific CTLs raises the important possibility that CTL responses may be able to control human tumors. It has become clear from animal and some human studies that immune responses to established tumors often do occur, with the clonal expansion of tumor-specific CTLs, but these responses are frequently weak and ineffective in eradicating the tumors. When the immunogenicity of these tumors is increased by ways discussed in this chapter, specific rejection of the tumors can be observed. Second, the diversity of rodent sarcoma tumor-specific transplantation antigens, and the identification

TABLE 18–1. Transplantation Antigens on Chemically and Virally Induced Tumors

	Treatment of Mouse			
Experiment	*Immunization with Killed Tumor Cells from*	*Challenge with Live Tumor Cells from*	*Result*	Conclusion
1	Chemically induced sarcoma A	Chemically induced sarcoma A	No growth	Immunity to chemically induced tumors is specific for individual tumors.
	Chemically induced sarcoma A	Chemically induced sarcoma B	Growth of chemically induced sarcoma B	
2	MSV-induced sarcoma A	MSV-induced sarcoma A	No growth	Immunity to virus-induced tumors is virus-specific.
	MSV-induced sarcoma A	MSV-induced sarcoma B	No growth of sarcoma B	
	MSV-induced sarcoma A	Chemically induced sarcoma C	Growth of chemically induced sarcoma C	
	MSV-induced sarcoma A	MuLV-induced sarcoma D	Growth of MuLV-induced sarcoma D	

Abbreviations: MSV, murine sarcoma virus; MuLV, murine leukemia virus.

of tumor antigens generated by *in vitro* mutagenesis, has established the potential for CTL surveillance of many different kinds of tumors.

We will now describe the characteristics of the different types of tumor antigens recognized by T lymphocytes, and we will return to effector responses to these antigens later in the chapter. The major categories of these antigens are listed in Table 18–2.

Products of Mutated Normal Cellular Genes Not Related to Oncogenesis

In vitro mutagenized mouse tumors were used for the first successful molecular genetic identification of tumor antigens recognized by T cells (see Box 18–1). These antigens turned out to be mutated versions of a variety of cellular proteins that are expressed in many cell types but have no known function or homologies. These proteins are apparently unrelated to the malignant phenotype, and the effects of the mutations, other than imparting immunogenicity, remain unknown. A similar phenomenon has been found in some human tumors. For example, a CTL clone derived from a melanoma patient recognizes a mutated peptide sequence of a protein of unknown function, the normal form of which is expressed in many tissues. The melanoma-generated peptide recognized by the CTL is abnormal in two respects, carrying both a point mutation and intronic sequences indicative of aberrantly spliced messenger RNA (mRNA). Although these findings confirm expectations based on the biology of tumors and our understanding of how T cell immunosurveillance works, the majority of human tumor antigens identified by recognition by specific CTLs are, surprisingly, not mutated in any way. This suggests that the high frequency of mutated proteins represented in the animal tumor models may be a function of the highly mutagenic protocols used to produce the tumors, but the importance of mutations in generating antigens expressed by human tumors may not be as great. In contrast, many of the human tumor antigens are products of aberrantly expressed but unmutated genes, as discussed later.

TABLE 18–2. Tumor Antigens That Stimulate T Cell Responses

Category	Examples
Tumor-specific transplantation antigens on chemically induced rodent sarcomas	No examples defined molecularly
Products of random point mutations in cellular genes not involved in tumor pathogenesis	p91A mutation in mutagenized murine mastocytoma MUM-1 melanoma antigen with a serine-to-isoleucine mutation in peptide recognized by melanoma-specific CTLs
Oncogene products	$p21^{ras}$ proteins with point mutation at position 12 (10% of human carcinomas) p210 product of *bcr-abl* rearrangements (chronic myelogenous leukemias) HER-2/*neu*, overexpressed normal gene (various carcinomas)
Mutated tumor-suppressor gene products	p53 (~50% of human tumors)
Products of silent genes normally not expressed in most tissues	MAGE 1, -3, BAGE, GAGE (expressed by many human melanomas, many types of carcinomas, normal testis and placenta)
Viral gene products in virus-associated malignancies	SV40 T antigen (SV40-induced rat tumors) Human papillomavirus E6 and E7 gene products (human cervical carcinoma) Epstein-Barr virus EBNA-1 gene product (Burkitt's, lymphoma, and nasopharyngeal carcinoma)
Products of tissue-specific genes expressed by normal cell types from which the tumor was derived	Tyrosinase, gp100, MART-1 (expressed exclusively by melanocytes and melanomas)

Abbreviations: SV, simian virus; EBNA, Epstein-Barr nuclear antigen.

Adapted from Pardoll, D. M. Cancer vaccines. Immunology Today 14:310–316, 1993.

Products of Oncogenes and Mutated Tumor Suppressor Genes

Virtually all tumors express genes whose products are required for malignant transformation or for maintenance of the malignant phenotype. In many instances, these genes have been identified, and often they are altered forms of normal cellular genes that control cell proliferation and differentiation. Such cellular proto-oncogenes may be altered by carcinogen-induced point mutations, deletions, or chromosomal translocations to form oncogenes whose products have transforming activity. In addition, viral integration into normal cellular proto-oncogenes can result in structurally abnormal products that have oncogenic activity. Tumor-suppressor genes also encode proteins required for normal cellular growth and differentiation, and mutations in these genes can result in nonfunctional products, leading to malignant transformation. The altered forms of proto-oncogene and tumor-suppressor gene products expressed in tumors should theoretically stimulate immune responses in the host, because these altered forms are not normally expressed as self proteins, and T cell tolerance to these abnormal proteins should not develop prior to their expression in tumors. These proteins are usually intracellular molecules that are likely to be processed and presented as peptides in association with class I MHC molecules, but phagocytosis or internalization of tumor cells or shed antigens may also result in class II MHC–associated presentation.

The mutations in some oncogenes are remarkably consistent among many different tumors, such as position 12 mutations in $p21^{ras}$ proteins, probably because only selected mutations impart a growth advantage to the cell. Furthermore, relatively few mutations appear in a wide variety of tumors. Because their amino acid sequences are known and they are widely distributed on many different tumors, oncogene or tumor-suppressor gene products are potential targets for cancer immunotherapy. It has been established that both human $CD4^+$ and $CD8^+$ T cells can respond to the products of some of these genes, including mutated Ras, p53, and *bcr-abl* proteins. However, there is very little evidence that these antigens induce protective T cell responses in humans. One oncogene called HER-2/*neu* is not a mutated gene but transforms cells when it is overexpressed. It encodes a membrane protein that is abundant on the cell surfaces of several different types of human carcinomas, and this protein does appear to be able to stimulate both $CD4^+$ helper T cell and $CD8^+$ CTL responses to these tumors. For example, CTLs that recognize a peptide derived from the HER-2/*neu* protein have been found among T cells specific for an ovarian carcinoma, and among $CD8^+$ T cells infiltrating breast and lung tumors. Clinical trials using this HER-2/*neu*–derived peptide as an immunogen to boost anti-tumor immunity are in progress.

PRODUCTS OF NORMALLY SILENT GENES

Some genes are usually not expressed in normal tissues or are expressed only early during development, before the mechanisms of self-tolerance are operative. When these genes are dysregulated as a consequence of malignant transformation of a cell and are expressed inappropriately in tumors, they may behave as tumor antigens and evoke immune responses. These tumor antigens may be shared by many different tumors. Mouse and human genes that encode these types of tumor antigens recognized by T cells have recently been identified using tumor antigen-specific CTL clones and DNA library transfections as described in Box 18–1. The functions of the proteins encoded by these genes are unknown, but they are not required for the malignant phenotype of the cells, and their sequences are identical to the corresponding genes in normal cells, i.e., they are not mutated. One of the mouse genes identified in this way is expressed on mast cell tumors and perhaps on some immature normal mast cells but not on other cells, and its protein product stimulates CTL-mediated tumor rejection *in vivo*. The MAGE (melanoma antigen) genes, first isolated from human melanoma cells, encode cellular protein antigens recognized by many different melanoma-specific CTL clones derived from different melanoma-bearing patients. There is a family of at least 12 homologous MAGE genes, but peptides from only two MAGE proteins are known to be recognized by melanoma-specific CTLs. MAGE proteins are variably expressed on many tumors in addition to melanomas, including carcinomas of the bladder, breast, skin, lung, and prostate, and some sarcomas. MAGE expression on normal tissues is restricted to testis and placenta, both of which are immunologically privileged sites that may not support the induction of tolerance. Subsequent to the identification of the MAGE genes, two other unrelated gene families have been identified, called BAGE and GAGE, which encode melanoma antigens recognized by autologous CTL clones derived from melanoma patients. Like the MAGE genes, the BAGE and GAGE genes are normally silent in most normal tissues, except testis, but they are expressed on a variety of malignant tumors. Although there is no evidence that expression of any of these genes induces a protective tumor rejection response, it is clear that melanoma patients do have memory CTLs that are specific for several MAGE, BAGE, and GAGE peptides. Therefore, there is great interest in attempting to boost the CTL response to these antigens in melanoma patients by the use of "therapeutic" vaccines.

TUMOR ANTIGENS ENCODED BY GENOMES OF ONCOGENIC VIRUSES

Both RNA and DNA viruses are implicated in the development of tumors in both experimental animals and humans. Virally induced tumors usually contain integrated proviral genomes in their cellular genomes and often express viral genome-encoded proteins. These endogenously synthesized proteins can be processed, and complexes of processed viral peptides with class I MHC molecules may be expressed on the tumor cell surfaces. Thus, tumor cells expressing viral proteins can stimulate and become the targets of specific T cell responses. Structurally and biologically distinct antigens are produced by various DNA and RNA tumor viruses.

DNA viruses are involved in the development of various tumors in experimental animals and humans. Papovaviruses (including polyoma virus and simian virus [SV]40) and adenoviruses induce malignant tumors in neonatal or immunodeficient adult rodents. Several genes in these viruses cooperate to cause malignant transformation of infected cells. In humans, DNA viruses are associated with the development of several different tumor types. For example, the Epstein-Barr virus (EBV) is associated with B cell lymphomas, Hodgkin's lymphoma, and nasopharyngeal carcinoma (Box 18–2). Human papillomavirus (HPV) is associated with most human cervical carcinomas. The viral genes responsible for producing the malignant phenotype in these human tumors are partially defined.

In most cases, DNA virus–induced tumor cells are latently infected with virus and do not produce viral particles. Virally encoded protein antigens that are not components of infectious viral particles may be found in the nucleus, cytoplasm, or

BOX 18-2. The Relationships Between Epstein-Barr Virus, Malignancy, and Immunodeficiency

Epstein-Barr virus (EBV) is a double-stranded DNA virus of the herpesvirus family. The virus is transmitted by saliva, infects nasopharyngeal epithelial cells and B lymphocytes, and is ubiquitous in human populations worldwide. It infects human B cells by binding specifically to the complement receptor type 2 (CR2), followed by receptor-mediated endocytosis. Two types of cellular infections can occur. In a lytic infection, viral DNA, RNA, and protein synthesis begin, followed by assembly of viral particles and lysis of the host cell. Alternatively, a latent, non-lytic infection can occur, in which the viral DNA is incorporated into the host genome indefinitely. Various virally encoded antigens are detectable in infected cells. **Epstein-Barr nuclear antigens (EBNAs)** include at least six nuclear proteins that are expressed early in lytic infections and may also be expressed by some latently infected cells. **Latent membrane protein (LMP)** is expressed on the surface of latently infected cells. Other viral structural protein antigens are expressed within infected cells and on released viral particles during lytic infections, including **viral capsid antigens (VCAs)**. Antibodies specific for VCAs are present in acutely infected, recovering, and remotely infected individuals.

EBV has profound effects on B lymphocyte growth characteristics *in vitro*. First, the virus is a potent, T cell–independent, polyclonal activator of B cell proliferation. Second, EBV can immortalize normal human B cells so that they will proliferate in culture indefinitely. The resulting long-term B lymphoblastoid cell lines are latently infected with the virus and may express EBNA proteins, but they do not have a malignant phenotype. The molecular basis for these effects of EBV on B cells is presently unknown, but both EBNA proteins and LMP have been implicated in growth-stimulation–transforming events. The binding of LMP receptor–associated factor (TRAF)-2 to the cytoplasmic tail of LMP is important for the effect of EBV on B cell proliferation (see Chapter 12, Box 12-5).

There is a wide spectrum of sequelae to infection by EBV. Most people are infected during childhood; they do not experience any symptoms and viral replication is apparently controlled by humoral and T cell–mediated immune responses. In previously uninfected young adults, infectious mononucleosis typically develops during EBV infection. This disease is characterized by sore throat, fever, and generalized lymphadenopathy. Large, morphologically atypical T cells are abundant in the peripheral blood of infectious mononucleosis patients. These cells are activated CTLs with specificity for EBV-encoded antigens. Previously infected, healthy individuals harbor the virus for the rest of their lives in latently infected B cells and, perhaps, in nasopharyngeal epithelium. An estimated 1 of every million B cells in a previously infected individual is latently infected. EBV infection is also strongly implicated as one of the etiologic factors for the development of certain malignancies, including nasopharyngeal carcinoma in Chinese populations, Burkitt's lymphoma in equatorial Africa, and histologically variable B cell lymphomas in immunosuppressed patients.

There is compelling evidence that T cell–mediated immunity is required for control of EBV infections and, in particular, for the killing of EBV-infected B cells. First, individuals with deficiencies in T cell–mediated immunity often have uncontrolled, widely disseminated, and perhaps lethal acute EBV infections. Second, EBV-infected B cells isolated from patients with infectious mononucleosis can be propagated *in vitro* indefinitely, but only if the patient's T cells are depleted or inactivated by drugs such as cyclosporin A. In fact, immortalization of normal peripheral blood B cells by *in vitro* infection with EBV is usually successful only if the donor's T cells are removed or inactivated. Third, CTLs specific for EBV-encoded antigens, including EBNAs and LMP, are present in acutely infected and completely recovered infectious mononucleosis patients. Cloned CTL lines have been established *in vitro* that specifically lyse EBV-infected B cells, and these CTLs most often recognize peptide fragments of EBNA and LMP proteins in association with class I MHC molecules. It is possible that EBV-specific T cells act *in vivo* to limit the polyclonal proliferation of infected B cells as well as to kill potentially immortalized clones of latently infected B cells. A loss of normal T cell–mediated immunity may allow latently infected B cells to progress toward malignant transformation. We discuss this hypothesis below.

The epidemiology and molecular genetics of Burkitt's lymphoma and other EBV-associated lymphomas have been the subject of intense investigation, and they offer fascinating insights into various aspects of viral oncogenesis and tumor immunity. Burkitt's lymphoma is a histologic type of malignant B cell tumor composed of monotonous, small, malignant B cells. The African form of the disease is endemic in regions where both EBV and malarial infection are common. In these regions, the tumor occurs frequently in young children, often beginning in the jaw. Virtually 100 per cent of African Burkitt's lymphoma patients have evidence of prior EBV infection, and their tumors almost all carry the EBV genome and express EBV-encoded antigens. Malarial infections in this population are known to cause T cell immunodeficiencies, and this may be the link between EBV infection and the development of lymphoma. Sporadic Burkitt's lymphoma occurs less frequently in other parts of the world, and although these B cell tumors are histologically similar to the endemic form, only approximately 20 per cent carry the EBV genome. Both endemic and sporadic Burkitt's lymphoma cells have reciprocal chromosomal translocations involving immunoglobulin gene loci and the cellular *myc* gene on chromosome 8 (Chapter 4; see Box 4-5).

B cell lymphomas occur at a high frequency in T cell–immunodeficient individuals, including individuals with congenital immunodeficiencies, AIDS patients, and kidney or heart allograft recipients receiving immunosuppressive drugs. Only some of these tumors can be called Burkitt's lymphomas, based on histologic appearance. Regardless of histologic appearance, many of these tumors are latently infected with EBV, like Burkitt's lymphoma. A smaller subset also contain *myc* translocations to Ig loci.

These observations can be synthesized into a hypothesis about the pathogenesis of EBV-associated B cell tumors. African children with malaria, allograft recipients, congenitally immunodeficient children, and AIDS patients all have deficiencies in normal T cell function. EBV infection proceeds unchecked in these individuals, and EBV-induced polyclonal proliferation of B cells is uncontrolled. This rapid, exuberant proliferation of B cells increases the chances of errors made during DNA replication, including translocations of oncogenes. The Ig loci are relatively accessible sites for translocations, compared with other loci in B cells. Translocation of the *myc* gene to the Ig locus leads to transcriptional deregulation and abnormal expression of *myc*, and this appears to be causally related to malignant transformation and outgrowth of a neoplastic clone of cells. Other genetic events can also lead to transformation after EBV-induced B cell proliferation, since many EBV-positive tumors do not have *myc* translocations, especially in allograft recipients and AIDS patients. In these cases, the proteins encoded by the integrated EBV genome may contribute to the malignant phenotype in EBV-positive lymphomas. This proposed scheme predicts that early in their course, EBV-associated B cell tumors may be polyclonal, since they arise from a polyclonally stimulated population of normal B cells. Later, one or a few clones may obtain selective growth advantages, perhaps because of deregulation of *myc* or other cellular or viral genes. As a result, the polyclonal proliferation evolves into a monoclonal or oligoclonal tumor. In fact, this has been shown to be the case by Southern blot analysis of Ig gene rearrangements in EBV-positive B cell tumors from immunosuppressed patients.

plasma membrane of the tumor cells. Specific immunity to DNA virus-encoded nuclear antigens protects against tumor development in animals. For example, SV40-induced tumors in mice express antigens that induce specific protective immunity against subsequent challenge with other SV40-induced tumors, but not against tumors induced by other viruses. Because these antigens are targets for tumor transplantation rejection, they are functionally defined as tumor transplantation antigens. These virally-encoded tumor antigens, however, are not unique for each tumor but are shared by all tumors induced by the same type of virus (see Table 18–1).

Although both humoral and T cell–mediated immune responses to DNA virus-encoded tumor antigens occur, only T cells specific for these antigens have been shown to mediate tumor rejection *in vivo*. One such virally encoded antigen that stimulates tumor rejection is the T antigen, a nuclear protein in SV40-transformed cells. The T antigen is required to produce the malignant phenotype, and it is not part of infectious virus particles. Immunization of experimental animals with SV40 virus induces protective immunity against the development of SV40-induced tumors, and this immunity is mediated by T antigen–specific class I MHC–restricted CTLs. Adenovirus-induced rodent tumors express a virally encoded protein called E1A, which is found largely in the nucleus and is the principal determinant of the transformed phenotype of the infected cells. E1A is not part of infectious adenovirus particles. When class I–restricted CTLs specific for a peptide derived from the E1A protein are adoptively transferred into mice with adenovirus-induced tumors, these CTLs kill the tumors (Fig. 18–4).

A protective role of the immune system in

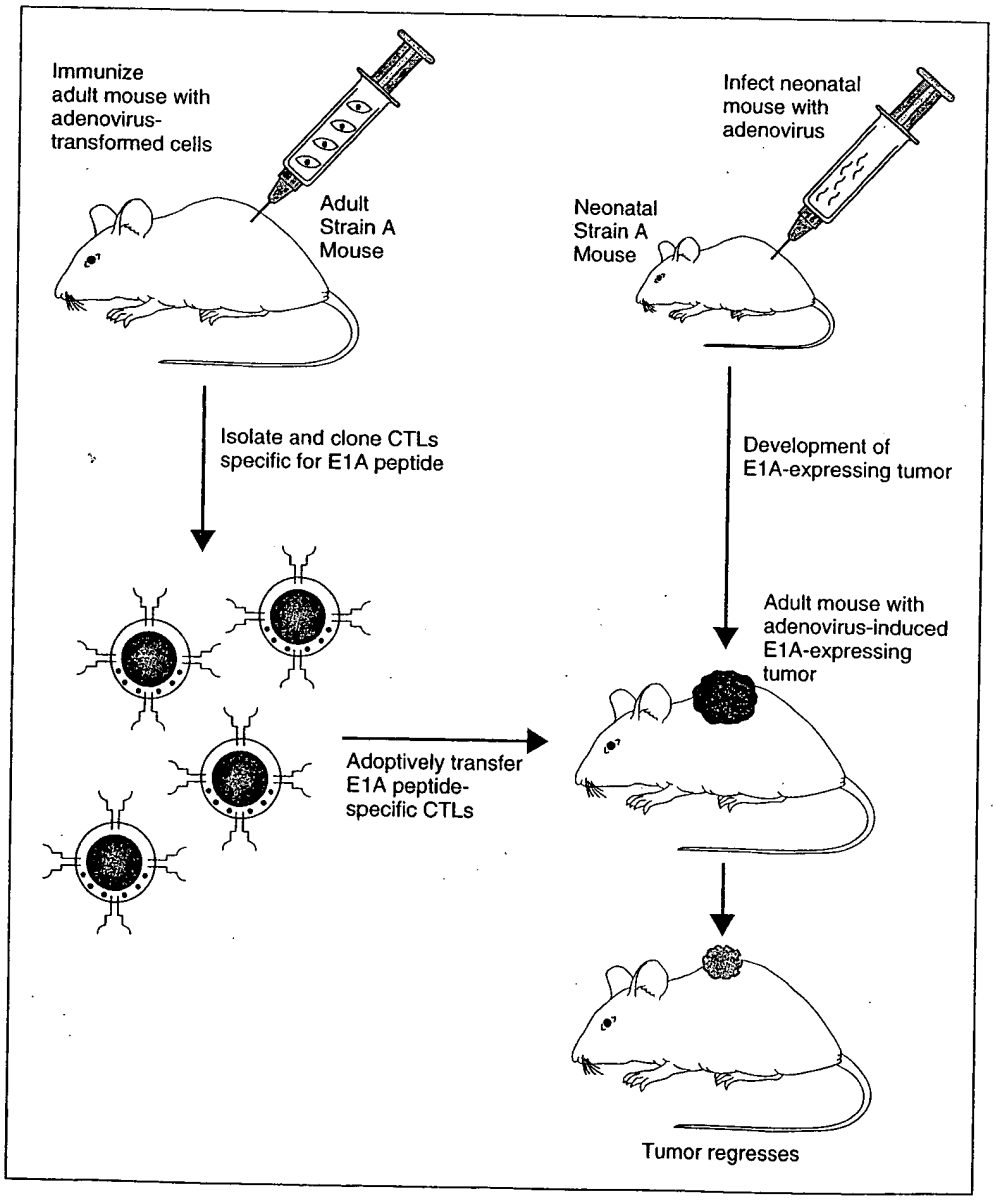


FIGURE 18–4. Viral antigen-specific cytolytic T lymphocytes (CTLs) kill virally infected tumors *in vivo*. If neonatal mice are infected with adenovirus, they develop malignant tumors as adults, and these tumors express the virally encoded E1A protein. The CTL clones isolated from a syngeneic mouse immunized with E1A-expressing cells can kill these E1A-expressing tumors when the CTLs are adoptively transferred to the tumor-bearing animal.

controlling the growth of DNA virus–induced tumors is suggested by the higher frequency of these tumors in immunodeficient individuals. In humans, EBV-associated lymphomas and HPV-associated skin cancers arise much more frequently in immunosuppressed individuals, such as allograft recipients receiving immunosuppressive therapy and acquired immunodeficiency syndrome (AIDS) patients, than in normal individuals. Adenovirus infection induces tumors much more frequently in neonatal or nude (congenitally T cell–deficient) mice, compared with normal adult mice. Thus, a competent immune system may play a role in tumor immunosurveillance because of its ability to recognize and kill virally infected cells. Although no DNA virus–encoded tumor antigen is known to induce protective immunity in human tumors, there is great interest in the products of two HPV genes, E6 and E7, which are constitutively expressed in cervical squamous cell carcinomas and are required for maintaining the malignant phenotype. Several E6- and E7-derived peptides bind to certain class I MHC alleles with high affinity, and they sensitize cells expressing these alleles for lysis by E6- or E7-specific CTL clones. Furthermore, at least one human cervical carcinoma cell line can be specifically lysed by these E6- and E7-specific CTLs. Accordingly, clinical trials of E6 and E7 peptide vaccines for treatment of HPV-positive cervical carcinoma have been started.

One of the clearest examples of viral oncogenesis is the development of tumors in animals infected with certain types of retroviruses (RNA tumor viruses). Some of these viruses carry well-defined oncogenes, induce tumors in days to weeks after infection, and are called **acute transforming retroviruses.** Examples of these acute transforming retroviruses include Rous sarcoma virus (carrying the *src* oncogene), avian myelocytomatosis virus (carrying the *myc* oncogene), and Kirsten murine sarcoma virus (carrying the K-*ras* oncogene). Other retroviruses, such as the murine leukemia viruses, cause tumors months after infection and do not carry any well-defined oncogenes. These slow-transforming retroviruses may cause tumors by inserting near, and dysregulating transcription of, cellular genes that are responsible for growth control and differentiation. The genomes of retroviruses are small, and they may express a limited number of potentially immunogenic proteins in their host tumor cells. These proteins include products of the envelope (*env*) gene, polymerase (*pol*) gene, core protein (*gag*) gene, and, in the case of acute transforming retroviruses, the oncogene. Retroviral oncogene products theoretically have the same potential antigenic properties as mutated cellular oncogenes, but they generally do not evoke strong immune responses *in vivo*. In contrast, humoral and cell-mediated immune responses to the *env* and *gag* products on tumor cells can be observed experimentally. Furthermore, *env* and *gag* products can stimulate CTL-mediated rejection of transplanted tumors. These antigens are shared by all tumors induced by the same type of retrovirus.

The only well-established human RNA tumor virus is human T cell lymphotropic virus-1 (HTLV-1), which is the etiologic agent of adult T cell leukemia/lymphoma (ATL), an aggressive malignant tumor of $CD4^+$ T cells. Although immune responses specific for HTLV-1 encoded antigens have been demonstrated, it is not clear whether they play any role in protective immunity against development of tumors in virally infected people. Furthermore, ATL patients are often profoundly immunosuppressed, perhaps because of an effect of the virus on $CD4^+$ T cells, which the virus preferentially infects.

TISSUE-SPECIFIC DIFFERENTIATION ANTIGENS RECOGNIZED BY TUMOR-SPECIFIC T CELLS

Tissue-specific differentiation antigens are proteins expressed by normal cells and are characteristic of a particular tissue type at a particular stage of normal differentiation of that tissue. Tumors arising from one type of tissue often express the differentiation antigens of that tissue. Since these antigens are part of normal cells, they would be expected to induce self-tolerance and not to stimulate immune responses against the tumors on which they are expressed. This is, in fact, the case for many such molecules, and they have been called tumor antigens only because they stimulate antibody responses in other species, as discussed later. It is surprising that the cloning of human melanoma antigens recognized by T cells from melanoma patients has turned up several tissue-specific antigens that are expressed by normal melanocytes and melanomas. The first such antigen identified was tyrosinase, an enzyme involved in melanin biosynthesis, which is expressed only in normal melanocytes and melanomas. Tyrosinase peptides eluted from melanoma cell class I MHC molecules sensitize targets for lysis by anti-melanoma CTLs. An interesting feature of at least one of the tyrosinase peptides eluted from melanoma cell MHC molecules is the presence of an aspartate residue that represents a post-translational modification of an asparagine residue encoded by the tyrosinase gene; the gene, however, is not mutated in the melanoma cells. Such modifications may be characteristic of tumor cells but not normal cells, reflecting the aberrant expression of modifying enzymes in the tumors, and they could explain why the product of a normal tissue-specific gene induces an immune response. Both class I MHC–restricted $CD8^+$ CTL clones and class II MHC–restricted $CD4^+$ T cell clones from melanoma patients recognize peptides derived from tyrosinase, raising the possibility that tyrosinase vaccines may stimulate both helper and cytolytic T cell responses to melanomas. MART-1 and gp100 or Pmel17 are two other proteins that are expressed

exclusively in melanocytes and melanomas and that are recognized by melanoma-specific CTLs. Both are transmembrane proteins of unknown function, and peptides derived from both are recognized by many independently derived melanoma-specific CTL lines. Adoptive transfer of gp100-specific T cells into melanoma patients is effective in reducing the tumor burden in some patients.

Tumor Antigens Recognized by Antibodies

Some molecules on tumor cell surfaces can elicit autologous antibody responses. In addition, some tumor molecules can be recognized by xenogeneic antibodies produced by immunizing an animal of one species with tumor cells from another species. Such molecules do not necessarily stimulate immune responses in the tumor host, but the antibodies that bind to them are potentially valuable in the diagnosis and therapy of tumors. Many cell surface molecules on tumors that have been identified by xenogeneic antibodies are called tumor antigens, but almost all of them are shared by different tumors arising from the same types of cells, and most, if not all, may also be found on some normal cells or benign tumor cells. Therefore, these molecules are tumor-associated antigens and not tumor-specific antigens. Most of these molecules do not stimulate immune responses in tumor hosts because they are normal self proteins, and they should be fully capable of inducing tolerance. It is surprising that there are cases in which antibodies and T cells specific for these tumor-associated antigens are found in tumor hosts, but there is no evidence that the antibody responses are protective. Several classes of these antigens exist, and many different ones may be expressed on the same tumor. We will consider some of the tumor antigens recognized by antibodies, dividing our discussion on the basis of biochemical characteristics or patterns of tissue distribution of these antigens.

Oncofetal Antigens

Oncofetal antigens are proteins normally expressed on developing (fetal) but not adult tissues. They are expressed on tumor cells as a result of the derepression of genes by unknown mechanisms. The importance of oncofetal antigens is that they provide markers that aid in tumor diagnosis. As techniques for detecting these antigens have improved, it has become clear that their expression in adults is not strictly limited to tumors. The proteins can be found in tissues in various inflammatory conditions, and even in small quantities in normal tissues. Furthermore, oncofetal antigens are not antigenic in the host, since they are expressed as self proteins during development. Nonetheless, the study of oncofetal antigens has proved useful for diagnostic purposes and has provided some insights into tumor biology. The two most thoroughly characterized oncofetal antigens are **carcinoembryonic antigen** (**CEA**, CD66) and **alpha-fetoprotein (AFP).**

CEA is a highly glycosylated 180 kD integral membrane protein that is a member of the Ig superfamily. CEA is also released into the extracellular fluid. Normally, high CEA expression is restricted to the gut, pancreas, and liver during the first two trimesters of gestation, and reduced expression is found in normal adult colonic mucosa and lactating breasts. CEA expression is greatly increased in colonic, pancreatic, gastric, and breast carcinomas, resulting in a rise in serum levels. Furthermore, post-translational processing of CEA may be altered in tumor cells. Serum CEA, detected by xenogeneic antibodies, is accordingly used to monitor the occurrence or recurrence of metastatic carcinoma after primary treatment. There is very little evidence for any significant anti-CEA humoral response in human cancer patients. Recent studies have demonstrated that CEA functions as an intercellular adhesion molecule, promoting the binding of tumor cells to one another. Thus, CEA may play a role in the way tumor cells interact with one another and with the tissues in which they are growing. The usefulness of CEA as a diagnostic marker for cancer is somewhat limited by the fact that serum CEA can also be elevated in the setting of non-neoplastic diseases such as chronic inflammatory conditions of the bowel or liver.

AFP is a 70 kD α-globulin glycoprotein normally synthesized and secreted in fetal life by the yolk sac and liver. Fetal serum concentrations can be as high as 2 to 3 mg/ml, but in adult life the protein is replaced by albumin, and only low levels are present in the serum. Serum levels of AFP can be significantly elevated in patients with hepatocellular carcinoma, germ cell tumors, and, occasionally, gastric and pancreatic cancers. An elevated serum AFP level is a useful indicator of advanced liver or germ cell tumors, or of recurrence of these tumors after treatment. Furthermore, the detection of AFP in tissue sections by immunohistochemical techniques can help in the pathologic identification of tumor cells. The diagnostic value of AFP as a tumor marker is limited by the fact that elevated serum levels are also found in non-neoplastic liver diseases, such as cirrhosis. As with CEA, there is little evidence that AFP-specific anti-tumor immune responses occur in cancer patients.

Altered Glycolipid and Glycoprotein Antigens

Most human and experimental tumors express higher levels of and/or abnormal forms of surface glycoproteins or glycolipids, including gangliosides, blood group antigens, and mucins. The abnormal forms are a result of alterations in the sequential addition of carbohydrate moieties to core protein or lipid molecules. Some aspects of the malignant phenotype of tumors, including tissue invasion and metastatic behavior, may in part be a function of

altered cell surface properties that result from abnormal glycolipid and glycoprotein synthesis. Many antibodies have been raised in animals that recognize carbohydrate groups or abnormally exposed peptide cores of these molecules. Although most of the epitopes recognized by these antibodies are not specifically expressed on tumors, there is a relative abundance on cancer cells. This class of TAAs continues to be a preferred target for antibody-based approaches to cancer therapy.

The gangliosides are neuraminic acid–containing glycosphingolipids, certain forms of which are expressed at particularly high levels on melanomas and some brain tumors compared with the normal cells from which these tumors are derived. For example, the gangliosides GM_2 and GD_2 are present at high density on the surface of melanomas and therefore are considered potential targets for immunotherapy. The tumor gangliosides have been studied with xenogeneic antibodies, but in addition, normal individuals and melanoma patients appear not to be tolerant to these molecules, since they make their own anti-ganglioside antibodies. In fact, there have been successful attempts at inducing high IgM antibody responses in melanoma patients by immunizing with GM_2 plus adjuvant vaccine, and this has correlated with prolonged clinical remission of the tumors.

Blood group antigens are carbohydrate epitopes on glycosphingolipid or glycoprotein molecules expressed on the surfaces of blood cells and epithelial cells (see Box 17–2, Chapter 17). Different epitopes are created depending on the expression of glycosyltransferases, and some of these antigens are aberrantly expressed in carcinomas, particularly the T and sialylated Tn antigens. Soluble forms of these antigens are being used as experimental cancer vaccines. These and other carbohydrate antigens are not likely to stimulate T cells required for isotype switching and memory B cell generation, and therefore their long-term usefulness as immunogens for cancer immunotherapy may be limited.

Mucins are high-molecular-weight proteoglycans containing numerous O-linked carbohydrate side chains on a core polypeptide. Because of their complicated branching structure, there is potential for great antigenic variability. Dysregulated expression in tumors of the numerous enzymes that synthesize these carbohydrate side chains can lead to the appearance of relatively tumor-specific epitopes, either on the carbohydrate side chains themselves or on the abnormally exposed polypeptide core. Several mucins have been the focus of diagnostic and therapeutic studies, including CA-125 and CA-19-9, expressed on ovarian carcinomas, and MUC-1, expressed on breast carcinomas. Unlike many mucins, MUC-1 is an integral membrane protein that is normally expressed only on the apical surface of breast ductal epithelium, a site that is relatively sequestered from the immune system. In ductal carcinomas of the breast, however, the molecule is expressed in an unpolarized fashion and contains new, highly tumor-specific carbohydrate and peptide epitopes detectable by mouse monoclonal antibodies. The peptide epitopes induce both antibody and T cell responses in cancer patients and are therefore being considered as candidates for anti-tumor vaccines.

TABLE 18–3. Examples of Tissue-Specific Tumor Antigens Used in Clinicopathologic Analysis of Tumors

Tissue of Origin	Tumor	Antigens
B lymphocytes	B cell leukemias and lymphomas	CD10 (CALLA) Immunoglobulin
T lymphocytes	T cell leukemias and lymphomas	Interleukin-2 receptor (α chain), T cell receptor, CD45R, $CD4^+/CD8^+$
Prostate	Prostatic carcinoma	Prostate-specific antigen Prostatic acid–phosphatase
Neural crest–derived	Melanomas	S-100
Epithelial cells	Carcinomas	Cytokeratins

Abbreviations: CALLA, common acute lymphoblastic leukemia antigen.

TISSUE-SPECIFIC DIFFERENTIATION ANTIGENS

We have discussed tissue-specific antigens recognized by T cells earlier in the chapter. The clinical significance of differentiation antigens on tumors relates to their use as targets for immunotherapy, and also as diagnostic markers of the tissue of origin of tumors. The histologic appearance of a tumor may not be characteristic enough to permit a diagnosis of the type of normal tissue from which the tumor arose. Therefore, antibody probes for the expression of tissue-specific antigens may be required. For example, lymphomas arising from the malignant transformation of developing B cells often may be diagnosed as B cell lineage tumors by the detection of a surface marker characteristic of normal pre-B cells, called CD10 (previously called common acute lymphoblastic leukemia antigen, or CALLA). Tumors arising from more mature B cells can be characterized by the presence of surface immunoglobulin. Examples of tissue-specific antigens expressed on tumors are listed in Table 18–3.

EFFECTOR MECHANISMS IN ANTI-TUMOR IMMUNITY

Both humoral and cell-mediated immune responses to tumor antigens have been demonstrated *in vivo,* and many immunologic effector mechanisms have been shown to kill tumor cells *in vitro.* The challenge for tumor immunologists is to determine which, if any, of these effector mecha-

nisms contributes to protective immune responses against human tumors and to enhance these effector mechanisms in ways that are relatively tumor specific. In this section, we briefly review the evidence for tumor killing by these various effector mechanisms and discuss which are the most likely to be relevant to human tumors.

T Lymphocytes

CTLs provide effective anti-tumor immunity *in vivo,* as demonstrated by the experimental tumor transplantation studies discussed earlier. In fact, CTL-mediated rejection of transplanted tumors is the only established example of completely effective specific anti-tumor immunity *in vivo*. In these cases, the effector cells are predominantly $CD8^+$ CTLs, which are phenotypically and functionally identical to the CTLs responsible for killing virus-infected or allogeneic cells described in Chapters 13 and 17. As discussed previously, CTLs may perform a surveillance function by recognizing and killing potentially malignant cells that express peptides which are derived from mutant cellular or oncogenic viral proteins and which are presented in association with class I MHC molecules.

The importance of this form of immunosurveillance for common, non-virally induced tumors is not established since such tumors do not arise more frequently in T cell–deficient animals or people, or in patients with drug- or virus-induced suppression of T cell immunity. On the other hand, as discussed earlier, tumor-specific CTLs can be isolated from animals and humans with already established tumors. For example, peripheral blood lymphocytes from patients with advanced carcinomas and melanomas contain CTLs that can lyse explanted tumors from the same patients. Furthermore, mononuclear cells derived from the inflammatory infiltrate in human solid tumors, called **tumor-infiltrating lymphocytes (TILs),** also include CTLs with the capacity to lyse the tumor from which they were derived. Although these CTL responses may not be effective in eradicating most tumors on their own, enhancement of CTL responses is a promising approach for anti-tumor therapy in the near future. CTL-mediated surveillance against cells infected with oncogenic viruses probably does occur naturally, as suggested by the fact that tumors associated with viral infections occur more frequently in immunosuppressed patients.

Although $CD4^+$ helper T cells are not generally cytotoxic to tumors, they may play a role in anti-tumor responses by providing cytokines for effective CTL development (see Chapter 13). In addition, helper T cells that are activated by tumor antigens may secrete tumor necrosis factor (TNF) and interferon-γ (IFN-γ), which can increase tumor cell class I MHC expression and sensitivity to lysis by CTLs. It is also possible that tumor-specific helper T cells may promote delayed type hypersensitivity (DTH) responses against tumors. A minority of tumors that express class II MHC molecules may directly activate tumor-specific $CD4^+$ helper T cells. More commonly, class II–expressing professional antigen-presenting cells (APCs) process and present internalized proteins derived from dying or phagocytosed tumor cells. $CD4^+$ helper T cells from some tumor-bearing individuals are specific for tumor antigens, including tyrosinase peptides and mutated Ras protein, but a thorough analysis of other antigens that tumor-specific helper T cells may recognize has not been accomplished.

Natural Killer Cells

NK cells may be effector cells of both innate and specific immune responses to tumors. NK cells can be activated by direct recognition of tumors, or as a consequence of cytokines produced by tumor-specific T lymphocytes. They use the same lytic mechanisms as CTLs to kill cells, but they do not express T cell antigen receptors, and they have a broad range of specificities (see Chapter 13). NK cells can lyse both virally infected cells and certain tumor cell lines, especially hematopoietic tumors, *in vitro*. In fact, lysis of such cell lines serves as the major bioassay for NK activity. There appears to be a degree of specificity to NK killing, since many virally infected cells or tumor cells and most normal cells are not susceptible to NK lysis *in vitro*. NK cell recognition of MHC molecules on a potential target inhibits NK cell lysis of that target (see Chapter 13). Thus, the down-regulation of MHC expression on many tumor cells, which may allow them to escape CTL lysis, makes them particularly good targets for NK cells. In addition, NK cells can be targeted to antibody-coated cells because they express low-affinity Fc receptors ($Fc\gamma RIII$ or CD16) for IgG molecules. The tumoricidal capacity of NK cells is increased by cytokines, including interferons, TNF, interleukin-2 (IL-2), and interleukin-12. Therefore, their role in anti-tumor immunity may depend on the concurrent stimulation of T cells and macrophages that produce these cytokines. There is great interest in the role of IL-2-activated NK cells in tumor killing. These cells, called **lymphokine-activated killer (LAK) cells,** are derived *in vitro* by culture of peripheral blood cells or tumor-infiltrating lymphocytes from tumor patients with high doses of IL-2 (see Chapter 13). LAK cells exhibit a markedly enhanced and nonspecific capacity to lyse other cells, including tumor cells. The use of LAK cells in adoptive immunotherapy of tumors will be discussed later.

A role for NK cells in tumor immunity *in vivo* is suggested by a variety of indirect evidence. For example, the incidence of tumors in different strains of inbred mice, or in mice of different ages, correlates inversely with the functional capacity of NK cells in these mice. It is interesting that T cell–deficient nude mice have normal or elevated num-

bers of NK cells, and they do not have a high incidence of spontaneous tumors. NK cells may play a role in immunosurveillance against developing tumors, especially those expressing viral antigens. However, a high level of NK activity is not present in the cellular infiltrates associated with solid human tumors, before *in vitro* expansion with IL-2.

Macrophages

Macrophages are potentially important cellular mediators of anti-tumor immunity. Their role is largely inferred from the demonstration that activated macrophages can preferentially lyse tumor cells, and not normal cells, *in vitro*. Like NK cells, macrophages express Fcγ receptors (high affinity FcγRI and FcγRIII), and they can be targeted to tumor cells coated with antibody. There are probably several mechanisms of macrophage killing of tumor target cells that are essentially the same as the mechanisms of macrophage killing of infectious organisms. These mechanisms include the release of lysosomal enzymes, reactive oxygen metabolites, and, in mice, nitric oxide.

Activated macrophages also produce the cytokine **tumor necrosis factor (TNF)**, which, as its name implies, was first characterized as an agent that can kill tumors but not normal cells. The various actions of TNF were discussed in Chapter 12. There is some evidence that TNF plays a role in macrophage-mediated tumor killing. For example, tumor cells selected *in vitro* for resistance to killing by TNF are often also resistant to killing by macrophages. Killing by both macrophages and TNF is slow (24 to 48 hours), can be augmented by protein or RNA synthesis inhibitors, and is due to apoptosis.

TNF kills tumors by direct toxic effects and indirectly by effects on tumor vasculature. Direct toxicity depends on binding of TNF to high-affinity cell surface receptors onto tumor cells. The toxicity is in part due to activation of a cell death pathway similar to that induced by Fas ligand binding to Fas (see Chapter 10). The toxicity may also be a result of the production of free radicals. Normal cells respond to TNF by synthesizing superoxide dismutase, an enzyme that participates in the inactivation of free radicals. In contrast, many tumor cells fail to make superoxide dismutase in response to TNF. Direct toxic effects of TNF may also involve disruption of cytoskeletal proteins, or interference with gap junction formation. TNF can indirectly cause tumor necrosis by inducing thrombosis in tumor vessels *in vivo*. This is suggested by the observations that even tumor cells lacking TNF receptors can be eradicated in mice by treatment with TNF, and that TNF selectively eradicates vascularized tumors and is much less effective in killing avascular tumors. Histologically, the response to TNF, described as hemorrhagic necrosis, looks very much like the localized Shwartzman reaction described in Chapter 12 (see Box 12–3). This resemblance has led to the suggestion that TNF acts selectively on tumor vessels to produce a Shwartzman-like reaction, causing thrombosis of the vessels and ischemic necrosis of tumors. Tumor vessels may be already "primed" to trigger the Shwartzman response once they encounter TNF. Some tumor-derived angiogenic factors, such as vascular endothelial growth factor, potentiate endothelial cell responses to TNF. These tumoricidal effects of TNF have been exploited in clinical practice, as we will discuss later.

Antibodies

Antibodies are probably less important than T cells in mediating effective anti-tumor immune responses, but, as we have discussed, tumor-bearing hosts do produce antibodies against various tumor antigens. In some instances, these antibody responses are specific for viral antigens. For example, patients with EBV-associated lymphomas have serum antibodies against EBV-encoded antigens expressed on the surface of their tumor cells. In other cases, human cancer patients produce antibodies against their own tumors that can be used for *in vitro* "autologous typing" to identify tumor antigens. In these cases, the antigens recognized are almost always present on normal tissues as well. No evidence exists for a protective role of such humoral responses against tumor development or growth. Hybridomas have been prepared from the B cells of tumor patients that produce monoclonal antibodies reactive with antigens on the patients' tumors. Again, these antibodies are not specific for antigens expressed exclusively on tumor cells. The potential for antibody-mediated destruction of tumor cells has largely been demonstrated *in vitro* and is attributable to complement activation, or to antibody-dependent cell-mediated cytotoxicity in which Fc receptor–bearing macrophages or NK cells mediate the killing. Whether or not these Ig-dependent mechanisms of tumor killing play a role *in vivo* remains unknown.

MECHANISMS OF EVASION OF THE IMMUNE SYSTEM BY TUMORS

Although malignant tumors may express protein antigens that are recognized as foreign by the tumor host, and although immunosurveillance may limit the outgrowth of some tumors, it is clear that the immune system often does not prevent the occurrence of lethal human cancers. The simplest explanation for this may be that the rapid growth and spread of a tumor overwhelms the effector mechanisms of immune responses. In addition, many tumors acquire the ability to evade immune responses, much like microbes. A major focus of tumor immunology is to understand the ways in which tumor cells may evade immune destruction, with the hope that interventions can be designed

to increase the immunogenicity of tumors or the responses of the host. The process of evasion, often called **tumor escape,** may be a result of several mechanisms.

1. Class I MHC expression can be downregulated on tumor cells so that they cannot form complexes of processed tumor antigen peptides and MHC molecules required for CTL recognition. There are clear demonstrations that increasing class I MHC expression on tumor cells results in increased susceptibility of these cells to CTL lysis *in vitro* and decreased tumorigenicity *in vivo* (Fig. 18–5). Furthermore, transfecting class I MHC genes into murine tumor cells often decreases their ability to form tumors when they are transplanted into healthy animals. Viruses have evolved ways to decrease class I MHC gene expression and assembly with peptides, thereby blocking the presentation of viral antigens to CTLs. Examples include the effects of adenovirus E1A on class I transcription, and herpes simplex virus inhibition of TAP function (see Chapters 6 and 16). These same mechanisms that inhibit surface expression of peptide–class I MHC complexes in infected normal cells may be operative in virally induced tumors. However, when the level of MHC expression on a broad range of experimental or human tumor cells is compared with the *in vivo* growth of those cells, no clear correlation exists. For example, metastatic tumors, which presumably have evaded immune attack, do not on the average express any fewer MHC proteins than non-metastatic tumors.

2. Even tumors that express peptide–class I MHC complexes that are recognized by host CTLs may fail to activate the CTLs for two reasons. First, because most human tumor cells do not express class II MHC molecules, they cannot directly activate tumor-specific $CD4^+$ helper T cells. Anti-tumor CTL activity is likely to be partly dependent on

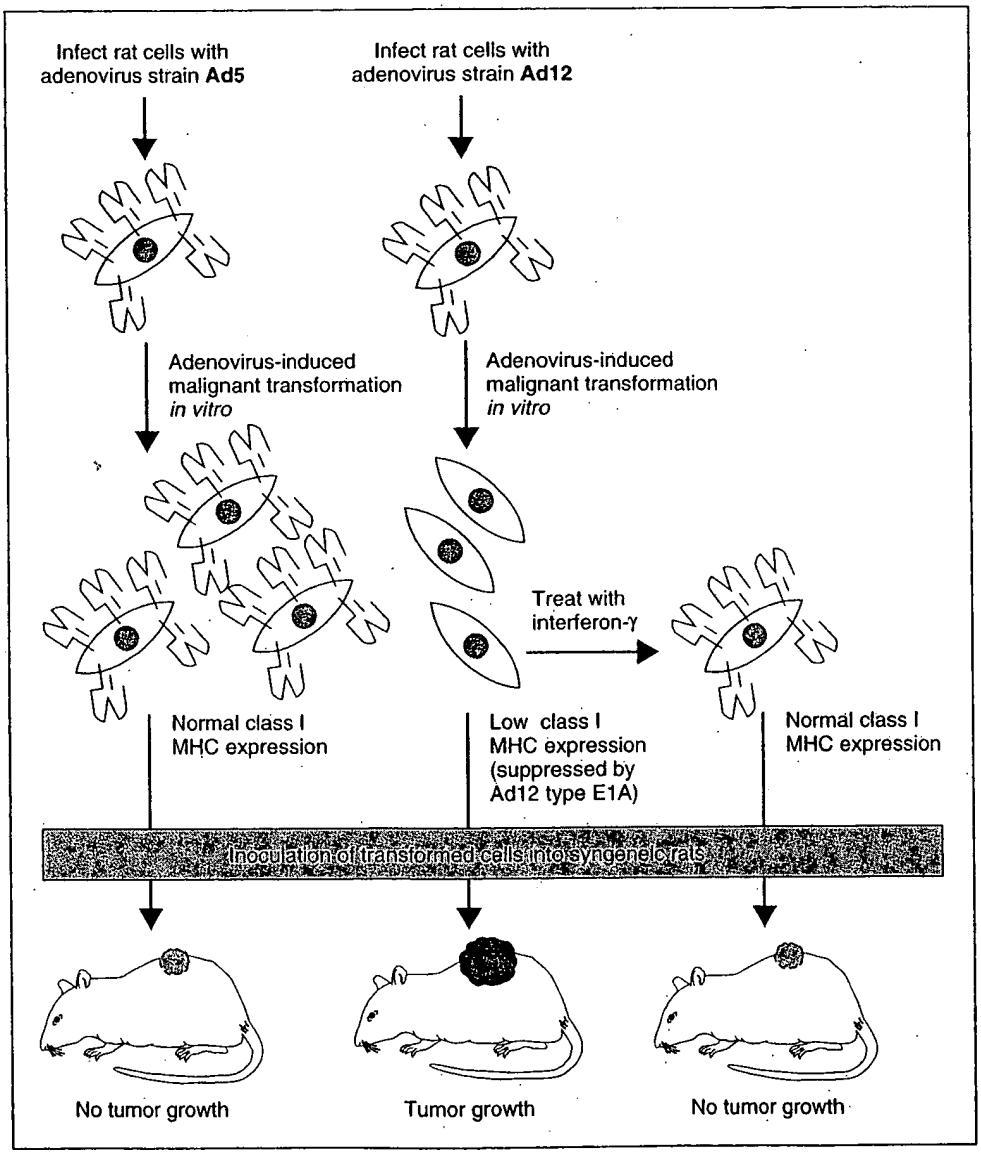


FIGURE 18–5. Relationship between class I MHC expression and tumorigenicity in adenovirus-induced tumors. Rat cells that are malignantly transformed *in vitro* by infection with the Ad5 strain of adenovirus express normal levels of class I MHC molecules and are not tumorigenic in syngeneic rats. In contrast, rat cells that are malignantly transformed *in vitro* by infection with the Ad12 strain of adenovirus express low levels of class I MHC molecules and are tumorigenic. Ad12-infected tumors can be induced to express higher levels of class I MHC molecules by interferon-γ, and this treatment renders them non-tumorigenic. The interpretation of this experiment is that class I MHC expression on a virally induced tumor permits the host animal to mount a protective immune response, presumably against a virally encoded antigen presented by the tumor cell in association with class I MHC molecules.

signals provided by helper T cells (see Chapter 13). If professional APCs do not adequately infiltrate these tumors, take up and present tumor antigens, and activate helper T cells, then maximal anti-tumor CTL differentiation will not occur. Second, a lack of costimulators on tumor cells may impair T cell activation. Most tumors are derived from tissues that do not express the costimulators that provide second signals for helper T cell activation (see Chapter 7). Furthermore, CTL activation also requires costimulation by cell surface molecules, such as B7-1 or B7-2, that are lacking on tumor cells. Tumor cell antigen presentation to T cells in the relative absence of costimulators may induce peripheral tolerance (clonal anergy) in tumor-specific T lymphocytes (see Chapter 10).

3. Tumor products may suppress anti-tumor immune responses. An example of an immunosuppressive tumor product is transforming growth factor-β (TGF-β), which is secreted in large quantities by many tumors and which inhibits a wide variety of lymphocyte and macrophage functions (see Chapter 12). Some tumors secrete IL-10, which also downregulates macrophage function.

4. A host may be tolerant to some tumor antigens. This may be because of neonatal exposure to such antigens or because the tumor cell may present its antigens to the immune system in a tolerogenic form. Neonatally induced tolerance has been demonstrated for tumors caused by the murine mammary tumor virus. This virus causes breast tumors in adult mice that have acquired the viral infection during neonatal life by nursing. Although these tumors are not seen as foreign in these mice and do not stimulate an immune response (because of neonatal tolerance), they are highly immunogenic when transplanted to syngeneic, virus-free adult mice. Another example of the relationship between neonatally induced tolerance and the growth of virally induced tumors is seen in mice that express the SV40 viral genome as a transgene. Transgenic mice that express the SV40 genes during early development have a high incidence of tumors, and this is correlated with tolerance to SV40 T antigen. In contrast, other SV40-transgenic mice in which expression of the transgene is delayed until later in life are not tolerant to the SV40 T antigen and have a low incidence of tumors.

5. Anti-tumor immunity can result in selection of mutant tumor cells that no longer express immunogenic peptide-MHC complexes. This could occur as a result of mutations or deletions in the genes encoding the tumor antigens, especially if the protein products of such genes are not critical for the malignant phenotype of the tumor. Alternatively, immunoselection may favor the growth of tumor cells with mutations or deletions in MHC genes needed for presentation of antigenic peptides. Given the generally high mitotic rate of tumor cells and their relative genetic instability, such mutations or deletions are theoretically likely. Analysis of tumors that are serially transplanted from one animal to another has shown that the loss of antigens recognized by tumor-specific CTL clones correlates with increased growth and metastatic potential.

6. The loss of surface expression of tumor antigens as a result of antibody binding, called antigenic modulation, leads to acquired resistance to immune effector mechanisms. Antigenic modulation is due to endocytosis or shedding of the antigen-antibody complexes. If antigenic modulation is caused by an anti-tumor antibody that does not fix complement, it may protect tumor cells from other complement-activating antibodies. Antigenic modulation is perhaps most relevant as a problem that complicates attempted passive immunotherapy with anti-tumor antibodies.

7. The kinetics of tumor growth may allow for the establishment of immunologically resistant tumors before an effective immune response develops. This phenomenon, called "sneaking through," can be experimentally modeled by transplantation studies. Transplantation of small numbers of tumor cells leads to establishment of lethal tumors (i.e., lack of rejection), whereas larger transplants of the same tumor are rejected. One presumed reason for this apparent contradiction is that small doses of tumor antigens are not sufficiently stimulatory to the immune system, and by the time many tumor cells grow in the transplant recipient, mutations in tumor antigen genes may have occurred that reduce the chance of immune recognition.

8. Tumor cell surface antigens can be hidden from the immune system by glycocalyx molecules, including sialic acid–containing mucopolysaccharides. This process is called antigen masking, and it may be a consequence of the fact that tumor cells often express more of these glycocalyx molecules than do normal cells. Similarly, some tumors may shield themselves from the immune system by activating the coagulation system, thereby investing themselves in a "fibrin cocoon."

IMMUNOTHERAPY OF TUMORS

The potential for treating cancer patients by immunologic approaches has held great promise for immunologists and cancer biologists over much of this century. Advances in our understanding of the immune system and in defining antigens on tumor cells that are targets of T cell immunity have encouraged many new strategies. Several of these approaches are aimed at augmenting weak host immune responses to tumor antigens. In this section, we describe some of the modes of tumor immunotherapy that have been tried in the past or are currently being investigated. The discussion is divided into sections on: induction and augmentation of active immune responses against an individual's tumor; and passive immunotherapy in which tumor-specific cells or antibodies raised *ex vivo* are administered to tumor patients.

Stimulation of Active Host Immune Responses to Tumors

NONSPECIFIC STIMULATION OF THE IMMUNE SYSTEM

Nonspecific immune stimulation of tumor patients with adjuvants, such as the bacille Calmette-Guérin (BCG) mycobacterium, injected at the sites of tumor growth has been tried for many years. This treatment serves mainly to activate macrophages and thereby promotes macrophage-mediated tumor cell killing. Oncologists are still assessing the potential of local BCG administration in bladder carcinomas and melanomas. An experimental approach to nonspecific immune stimulation is the administration of low doses of anti-CD3 antibodies to mice with transplanted fibrosarcomas. This treatment results in polyclonal activation of T cells and, consequently, prevention of tumor growth. Cytokine therapies, discussed below, represent another method of enhancing immune responses in a nonspecific manner.

VACCINATION WITH KILLED TUMOR CELLS

Induction of protective immunity to tumors can theoretically be accomplished by active immunization procedures with purified tumor antigens or cells expressing these antigens. One method is to inject killed or irradiated tumor cells together with nonspecific adjuvants. The rationale for this approach is that antigen-bearing tumor cells may be able to induce a strong immune response if they are delivered to the immune system under conditions that favor lymphocyte activation. Memory T cells expanded by such immunizations, it is hoped, would limit the growth of already established tumors. A wide variety of protocols has been tried, but the efforts have been largely unsuccessful, probably because the tumor cell vaccines do not effectively activate specific CTL responses. A variation on this approach, which we will discuss later, is immunization with live tumor cells that have been transfected with genes that render the cells more immunogenic.

VACCINATION WITH TUMOR ANTIGENS OR PEPTIDES

The identification of peptides recognized by human tumor-specific CTLs, and the cloning of genes that encode tumor-specific antigens recognized by CTLs, described earlier, has provided the basis for another form of active anti-tumor immunization, namely the introduction of specific tumor antigens into tumor-bearing patients using immunogenic vaccine preparations. The rationale for this "therapeutic" vaccine approach is the likelihood that the tumor-specific memory T cells in tumor patients could be actively and specifically stimulated by such vaccines, and further development of memory T cells could be enhanced. Immunization with these antigens can be theoretically accomplished by direct administration of purified peptide or protein, by immunization with cells expressing recombinant genes which encode these antigens, or by injection of expression vectors encoding tumor antigens (DNA vaccines). One drawback of peptide vaccines is that the administered tumor peptides are unlikely to efficiently replace other non-immunogenic peptides already bound to MHC molecules on tumor cell surfaces. Intact proteins, on the other hand, are most likely to enter the class II MHC pathway of antigen presentation, and to activate $CD4^+$ helper T cells, but not $CD8^+$ CTLs. DNA vaccines may be the best way to induce CTL responses because the DNA can be taken up by host cells and the encoded antigens will be translated in the cytoplasm and enter the class I MHC pathway of antigen presentation. For unique tumor antigens, such as may occur with random point mutations in cellular genes, such immunization protocols may be impractical because they would require initial identification of these antigens from individual tumors by use of T cell probes. On the other hand, tumor antigens shared by many tumors, such as the MAGE, tyrosinase, and gp100 antigens on melanomas or position 12-mutated Ras proteins, are potentially useful immunogens for many different cancer patients. It would be possible to determine whether a patient's tumor expresses a particular common tumor antigen by use of polymerase chain reaction (PCR)-based detection of the relevant mutated gene, and human leukocyte antigen (HLA) typing could be used to determine whether the patient expresses MHC molecules that bind the immunodominant peptide from that antigen. As we have mentioned earlier in the chapter, vaccine trials are already under way for a variety of tumors.

Immunization with cells expressing high levels of tumor antigen genes has the advantage that such antigens will have ready access to class I MHC pathways of antigen presentation and may be more efficiently presented to CTLs than will antigens in cell-free vaccines. This process could be accomplished by linking the genes encoding the antigens to active promoters, transfecting the constructs into host tumor cells or other cell types *ex vivo*, and reintroducing the transfected cells into the patient. Alternatively, recombinant vaccinia virus vectors with tumor antigen gene inserts can be used to achieve expression of tumor antigens in the patient. Infection of animals with recombinant vaccinia virus–containing tumor antigen genes has already been shown to establish protective immunity to subsequent challenges with tumors expressing those antigens, but this method is not yet approved for humans.

The development of virally induced tumors can be blocked by vaccination with viral antigens or attenuated live virus. This approach has been successful in reducing the incidence of feline leukemia virus–induced hematologic malignancies in cats and in preventing the herpesvirus-induced lymphoma called Marek's disease in chickens. In

humans, the ongoing vaccination program against the hepatitis B virus (HBV) may reduce the incidence of hepatocellular carcinoma, a cancer associated with HBV infection of the liver.

AUGMENTATION OF HOST IMMUNITY TO TUMORS WITH COSTIMULATORS AND CYTOKINES

As we have discussed previously in this chapter, tumor cells may induce weak immune responses because they lack costimulators, and usually they do not express class II MHC molecules, so they do not activate helper T cells. Two potential approaches for boosting host responses to tumors are to provide costimulation for tumor-specific T cells artificially, and to provide exogenous cytokines that can enhance T cell growth and activation, thus replacing helper T cell functions. Many cytokines also have the potential to enhance nonspecific inflammatory responses which by themselves may have anti-tumor activity.

The efficacy of enhancing T cell costimulation for anti-tumor immunotherapy has been demonstrated by animal experiments in which tumor cells were transfected with genes that encode costimulatory molecules (see Chapter 7). For example, tumor cells transfected with the gene encoding the B7-1 costimulatory molecule are potent stimulators of anti-tumor immune responses, compared with unmodified tumor cells (Fig. 18–6). When injected into naive syngeneic hosts, these B7-1–expressing tumor cells are rejected whereas unmodified tumor cells are not. In addition, injection of B7-1–expressing tumors at one site induces protective immunity against unmodified tumor cells injected later at a distant site. The reason for this probably is that B7-1 molecules enhance specific CTL responses, but the cytolytic activity of fully differentiated (effector) CTLs is not dependent on B7-1. These successes with experimental tumor models may lead to therapeutic trials in which a sample of a patient's tumor is propagated *in vitro,*

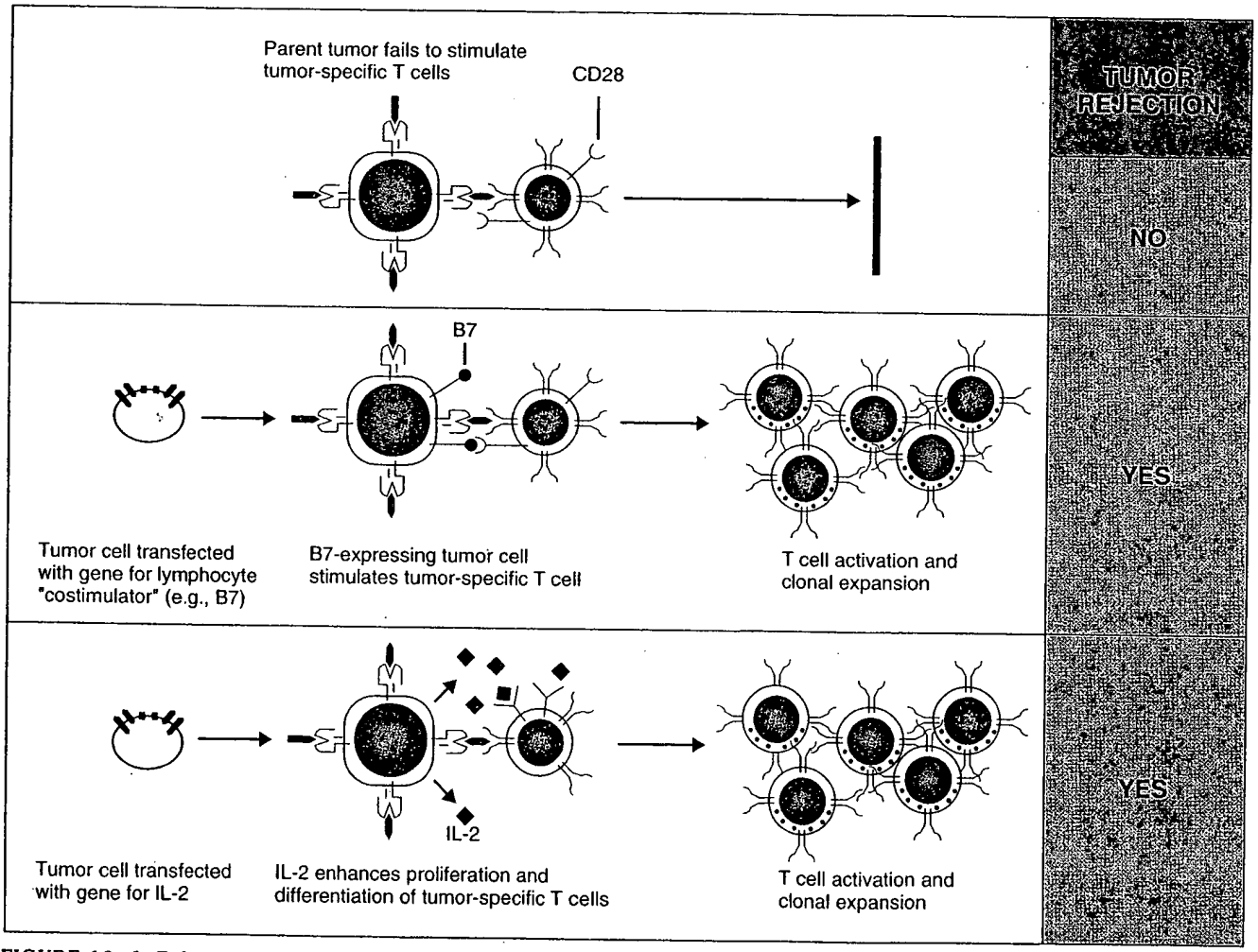


FIGURE 18–6. Enhancement of tumor cell immunogenicity by transfection of costimulator or cytokine genes. Tumor cells that do not adequately stimulate T cells when transplanted into an animal will not be rejected and therefore will grow into tumors. Transfection of these tumor cells with constitutively active genes encoding costimulators or cytokines can lead to enhanced immunogenicity of the tumors, T cell–mediated rejection, and therefore no tumor growth. In addition, after rejection of these modified tumor cells, the animals retain specific immunity to subsequent challenges by the parent (untransfected) tumor cells.

transfected with costimulator genes, irradiated, and reintroduced into the patient. Such approaches may succeed even if the rejection antigens expressed on tumors are not defined.

The potential of various cytokines to enhance both specific and innate immune responses against tumors has also been demonstrated in experimental models and has been realized in clinical practice. In several experimental protocols, tumor cells are transfected with cytokine genes in order to localize the cytokine effects to where they are needed. For instance, when rodent tumors transfected with IL-2, IL-4, IFN-γ, or granulocyte-macrophage colony–stimulating factor (GM-CSF) genes are injected into animals, the tumors are rejected or regress. In some cases, intense inflammatory infiltrates accumulate around the cytokine-secreting tumors, and the nature of the infiltrate varies with the cytokine (Table 18–4). Eosinophils and macrophages accumulate around IL-4–producing tumors, macrophages dominate infiltrates around IFN-γ–secreting tumors, and IL-2–producing tumors are surrounded by massive lymphocytic infiltrates. In essence, the cytokines elaborated by the transfected tumor cells are acting as adjuvants. The importance of these findings is that the type of inflammatory cells recruited by different cytokines may provide different effector functions as well as accessory cell functions required for optimal activation of T cells. An important fact is that, in several of these studies, the injection of cytokine-secreting tumors induced specific, T cell–dependent immunity to subsequent challenges by unmodified tumor cells. This is most evident with tumors expressing transfected GM-CSF genes, and it may reflect the ability of GM-CSF to induce the differentiation of immature dendritic cells into mature APCs. Thus, the local production of cytokines may augment specific T cell responses to tumor antigens, and cytokine-expressing tumors may act as true tumor vaccines. Several clinical trials with cytokine gene–transfected autologous or allogeneic tumors are under way in advanced cancer patients. Another approach to achieve cytokine production around tumor cells is the use of biodegradable polymer microspheres that release encapsulated cytokines gradually over extended time periods. The microspheres are mixed with irradiated tumor cells from a patient *ex vivo,* and the mixture is then reinjected. This method avoids the technically cumbersome requirement of transfecting tumor cells.

Cytokines are also administered systemically for the treatment of various human tumors. This type of experimental therapy became feasible when highly purified or recombinant cytokines were made available in sufficient quantities. Many of the protocols for cytokine therapy are continually being modified to take into account toxic systemic effects, and the application of this approach to different tumors is continually being explored.

1. IL-2, administered in high doses, is used alone or in conjunction with adoptive cellular immunotherapy (discussed later). After administration of IL-2, there is an increased number of blood lymphocytes and NK cells, an increase in NK and LAK cell activity, and increases in serum TNF, IL-1, and IFN-γ. Presumably, the IL-2 works by stimulating the anti-tumor activity of NK cells and/or CTLs, i.e., by inducing LAK cell differentiation *in vivo.* The treatment can be highly toxic, causing fever, pulmonary edema, and often shock. These effects occur because the IL-2 stimulates production of other cytokines by T cells, which have deleterious effects at high doses. IL-2 has been effective in inducing measurable tumor regression responses in about 10 to 15 per cent of patients with advanced melanoma and renal cell carcinoma and is currently an approved treatment for these cancers.

TABLE 18–4. Modification of Mouse Tumors by Transfected Cytokine Genes

Cytokine	Enhanced Rejection of Transfected Tumor	Inflammatory Infiltrate	Distant Immunity Against Parental Tumor
Interleukin-2	Yes (dependent on $CD8^+$ and not $CD4^+$ T cells)	Lymphocytes	Sometimes
Interleukin-4	Yes	Macrophages and eosinophils	Sometimes (dependent on both $CD8^+$ and $CD4^+$ T cells)
Interferon-γ	Sometimes (varies from tumor to tumor)	Variable	Sometimes
Tumor necrosis factor	Sometimes	Mixed neutrophils and lymphocytes	No
GM-CSF	Yes	Immature mononuclear cells	Yes (long-lived immunity; dependent on both $CD8^+$ and $CD4^+$ T cells)
Monocyte chemotactic protein-1	No	Macrophages	No
Interleukin 3	Sometimes	Immature mononuclear cells	Sometimes (long-lived immunity; dependent on both $CD8^+$ and $CD4^+$ T cells)

Abbreviations: GM-CSF, granulocyte-macrophage colony–stimulating factor.
Adapted from Pardoll, D. M. Cancer vaccines. Immunology Today 14:310–316, 1993.

2. TNF clearly has potent anti-tumor effects *in vitro*, and clinical trials of TNF in advanced cancer patients have been performed. Unfortunately, high doses of TNF produce many undesirable pathologic effects (see Chapter 12), and TNF can be highly toxic at the doses required for tumor killing *in vivo*. Local injection of TNF is used to treat limb sarcomas, and in these cases, venous return from the extremity is reduced during the treatment to minimize systemic effects.

3. Alpha-interferon (IFN-α) is a type I interferon, produced largely by leukocytes (see Chapter 12). It has antiproliferative effects on cells *in vitro*, increases the lytic potential of NK cells, and increases class I MHC expression on various cell types. Clinical trials of this cytokine indicate that it can induce regression of renal carcinomas, melanomas, Kaposi sarcomas, various lymphomas, and hairy cell leukemias (a B cell lineage tumor). In fact, IFN-α treatment of hairy cell leukemia was used routinely in many medical centers until a new and successful chemotherapeutic drug was recently introduced. Currently, IFN-α is used in conjunction with chemotherapy for the treatment of metastatic melanoma.

4. IFN-γ treatment of various hematopoietic and solid tumors has been intermittently successful, and intraperitoneal administration of IFN-γ for the treatment of ovarian carcinomas has also been evaluated, but this cytokine is not part of any established therapeutic regimen. The rationale for using IFN-γ is that the macrophage- and NK-activating properties of this cytokine, as well as its ability to upregulate MHC molecule expression, would help enhance anti-tumor immunity.

5. Hematopoietic growth factors, including GM-CSF and granulocyte colony–stimulating factor (G-CSF), are used in cancer treatment protocols, although not strictly to enhance immune responses against tumors. Rather, they shorten periods of neutropenia after chemotherapy or autologous bone marrow transplantation by stimulating maturation of granulocyte precursors.

6. There is a lot of interest in the potential of IL-12 to enhance anti-tumor NK- and T cell–mediated immune responses, and toxicity trials in advanced cancer patients are now under way.

Passive Tumor Immunotherapy

Passive immunotherapy involves the transfer of immune effectors into a patient from an external source. Antisera specific for bacterial or snake toxins are examples of passive therapy that are widely used in clinical practice. The same approaches have been used in tumor immunotherapy, with variable success, and have included passive transfer of lymphocytes and antibodies.

Adoptive Cellular Immunotherapy

Adoptive cellular immunotherapy refers to the transfer of cultured immune cells that have anti-tumor reactivity into a tumor-bearing host. Two variations to this approach have been tested in clinical trials.

1. **Lymphokine-activated killer (LAK) cell therapy** involves the *in vitro* generation of LAK cells by removing peripheral blood leukocytes from tumor patients and culturing the cells in high concentrations of IL-2. The LAK cells are then injected back into the cancer patients. As discussed previously, LAK cells are predominantly derived from NK cells. Adoptive therapy with autologous LAK cells, in conjunction with *in vivo* administration of IL-2 or chemotherapeutic drugs, has yielded impressive results in mice, with regression of solid tumors. Human LAK cell therapy trials have so far been largely restricted to advanced cases of metastatic tumors, and the efficacy of this approach appears to be highly variable from patient to patient.

2. **Tumor-infiltrating lymphocyte (TIL) therapy** involves the generation of LAK cells from mononuclear cells originally isolated from the inflammatory infiltrate present in and around solid tumors, obtained from surgical resection specimens. The rationale for this approach is that TILs may be enriched for tumor-specific CTLs and NK cells. In fact, TILs do include activated NK cells and CTLs, but only some of the cells in these mixed populations of cells are specific for the tumors from which they were isolated. As we have discussed previously, many melanoma-specific CTL clones have been derived from melanoma TILs. Human trials with TIL therapy are ongoing. One approach for local delivery of cytokines to tumors is transfection of TILs with cytokine genes; this has been attempted with TNF in a small number of patients with limited success.

Therapy with Anti-Tumor Antibodies

Many variations on the use of passively administered antibodies in cancer therapy have been tried. One approach is to use antibodies that bind tumor antigens as vehicles to bring toxic agents to, and selectively kill, tumor cells. The potential of using antibodies as "magic bullets" has been alluring to investigators for many years and is still a very active area of research. In addition, *in vivo* administration of antibodies specific for T cells may be employed to augment cellular responses nonselectively or to target immune effector cells to tumors. Several types of these antibody treatments are described below.

1. Anti-tumor antibodies coupled to toxic molecules, radioisotopes, and drugs have all been used in immunotherapy trials in cancer patients or in experimental animals. Toxins such as ricin or diphtheria toxin are highly potent inhibitors of protein synthesis and can be useful at extremely low doses if they are bound to antibodies to form **immunotoxins.** This approach requires the co-

valent attachment of the toxin (lacking its cell-binding component) to an anti-tumor antibody molecule without loss of toxicity or antibody specificity. The systemically injected immunotoxin must be endocytosed by tumor cells and delivered to the appropriate intracellular site of action. Another approach is to covalently attach antineoplastic drugs or cytocidal radioisotopes to antitumor antibodies.

Several practical difficulties must be overcome for this technique to be successful. The specificity of the antibody must be such that it does not significantly bind to non-tumor cells. As we have discussed, there are few truly tumor-specific antigens to select when an antibody-based immunotherapy approach is designed. Many TSAs are peptides derived from cytoplasmic proteins that are presented bound to class I MHC molecules, and antibodies that will recognize these bound peptides are difficult to make. Most anti-tumor antibodies are directed at cell surface TAAs that are more highly expressed on tumor cells than on normal tissues. It is also difficult to ensure that a sufficient amount of antibody reaches the appropriate tumor target before it is cleared from the blood by Fc receptor–bearing phagocytic cells. Such clearance may not only reduce anti-tumor effectiveness but may also damage phagocytic cells. The toxins, drugs, or radioisotopes attached to the antibody may have systemic effects as the result of circulation through normal tissues. For example, hepatotoxicity and vascular leak syndromes are common problems with immunotoxin reagents. Since the anti-human tumor antibodies used in clinical trials are usually made in other species, as are conjugated plant or bacterial toxins, there is frequently an immune response resulting in anti-antibodies or anti-toxins that may cause increased clearance rates or block binding of the therapeutic reagent to its target. One way to diminish this problem is to use recombinant, "humanized" antibodies comprising the variable regions of a mouse monoclonal antibody specific for the tumor antigen combined with human Fc portions (see Chapter 3, Box 3–1). Another problem with the use of anti-tumor antibodies is the outgrowth of mutant tumor cells that no longer express the antigens that the antibody recognizes. This is particularly likely to happen if the target antigens are not required for the malignant phenotype. One way to avoid this problem is to use cocktails of antibodies with specificities for different TAAs expressed on the same tumor.

The results of clinical trials with anti-tumor antibody conjugates (immunotoxins) have been variable. Toxin- and radionuclide-conjugated antibodies with specificities for various TAAs on melanomas and carcinomas have been tried. In addition, antibody conjugates specific for CD19, CD22, and CD30 have been used to treat B cell lymphomas. Several clinical trials have used antibodies specific for the human interleukin-2 receptor α (IL-2Rα) chain for treatment of adult T cell leukemias, which usually express high levels of IL-2Rα. Mouse and humanized anti-IL-2Rα antibodies have been used in unconjugated forms. Such antibodies could cause complement-mediated lysis of IL-2R–expressing tumor cells. Anti-IL-2 receptor antibodies have also been conjugated to various agents, including diphtheria toxin and the α-particle-emitting radionuclide yttrium 90. In a related strategy, a chimeric protein in which IL-2 itself is linked to the effector chain of *Pseudomonas* toxin has been used to treat T cell lymphomas. Anti-IL-2R therapy is not tumor-specific and may be immunosuppressive because normal, activated T cells would be rendered nonfunctional. In general, the efficacy of these various agents is limited, and only a small percentage of patients show significant reductions in tumor burden. Nonetheless, intermittent successes have encouraged further refinement of the reagents. Furthermore, trials of these reagents in immunodeficient mice with xenografted human tumors continue to generate new candidates for human trials. For example, a monoclonal antibody specific for a Lewis Y–related antigen conjugated to the chemotherapeutic agent doxorubicin cures widely metastatic human carcinomas transplanted into athymic mice.

2. Anti-idiotypic antibodies have been used in the treatment of B cell lymphomas that express surface Ig with particular idiotypes. The idiotype is a highly specific tumor antigen since it is expressed only on the neoplastic clone of B cells, and there was once great hope that anti-idiotypic antibodies would be effective therapeutic reagents with absolute tumor specificity. (Anti-idiotypic antibodies are raised by immunizing rabbits with a patient's B cell tumor and depleting the serum of reactivity against all other human immunoglobulins.) This strategy relies on complement fixation or antibody-dependent cell-mediated cytotoxicity (ADCC) to kill the lymphoma cells. The approach has not proved generally successful, and there are many reasons why it may not work. Because surface Ig expression is not functionally related to the malignant phenotype of the cell, selective outgrowth of non–Ig-expressing tumor cells can occur. Moreover, the high degree of somatic mutation known to occur in Ig genes results in the selective outgrowth of tumor cells with altered idiotypes no longer reactive with the anti-idiotypic antibody. Attempts to circumvent these problems with cocktails of several different antibodies have also not proved successful. More recently, immunization of animals with DNA encoding Ig idiotypes has been shown to induce anti-idiotypic antibody responses, and this strategy is being considered for the treatment of lymphomas.

3. Heteroconjugate antibodies may allow targeting of cytotoxic effector cells onto tumor cells. In this approach, an antibody specific for a tumor antigen is covalently coupled to an antibody directed against a surface protein on cytotoxic effec-

tor cells, such as NK cells or CTLs. Such heteroconjugates can promote binding of the effector cells to tumor cells. A heteroconjugate consisting of an anti-CD3 antibody coupled to an antibody against a tumor cell surface protein has been used to enhance CTL-mediated lysis of the tumor cell. In this case, the anti-CD3 antibody not only served to bring the CTL into contact with the target cell, but it also activated the CTL. A related approach is to use conjugates of antibodies specific for effector cells with hormones whose receptors are expressed on tumor cells. For example, anti-CD3 antibodies coupled to melanocyte-stimulating hormone enhance *in vitro* destruction of human melanoma cells by CTLs. These types of antibody therapies have so far been tried only in experimental animal studies.

Another way anti-tumor antibodies have been used for cancer treatment is for the *in vitro* depletion of bone marrow tumor cells by antibody plus complement-mediated lysis. This is useful for autologous bone marrow transplants in B cell lymphoma patients. In this protocol, some of the patient's bone marrow is removed, and the patient is given lethal doses of radiation and chemotherapy, which destroy tumor cells and the remaining normal marrow cells in the patient. The bone marrow removed from the patient is then treated with antibodies directed against B lymphocyte-specific antigens, which are known to be expressed on the B cell–derived lymphoma cells. Complement is then added to promote lysis of the lymphoma cells that have bound antibody. The treated marrow, having been purged of lymphoma cells, is transplanted back into the patient and can reconstitute the hematopoietic system destroyed by irradiation and chemotherapy.

SUMMARY

Malignant tumors express antigens that may stimulate and serve as targets for anti-tumor immunity. Protective anti-tumor immune responses occur in experimental animal models. However, it has been more difficult to demonstrate that innate or specific immune responses can protect humans against tumor growth. The development of virally induced tumors, which express virally encoded antigens, can be inhibited by specific immune responses. Antigens unique to individual tumors, which stimulate specific rejection of transplanted tumors, do occur in experimental animal tumors. Other tumor antigens that can stimulate immune responses are shared by different tumors, and these include viral antigens, products of mutated or rearranged oncogenes or tumor suppressor genes, and products of derepressed genes. Human CTL clones specific for tumor antigens have been isolated from tumor patients, and the peptide antigens or genes encoding these antigens have been identified. Tumors may also express tissue differentiation antigens or embryonic antigens to which the host is tolerant; these molecules are useful diagnostic markers. Many immunologic effector mechanisms can destroy tumor cells *in vitro*. One or more of these mechanisms may work on tumor cells *in vivo*, and different mechanisms may be effective on different tumors. CTLs are probably the most important effectors of anti-tumor immunity *in vivo*, although NK cells and macrophages may also be involved. Various mechanisms may explain how antigen-expressing tumors escape destruction by the immune system. These mechanisms include poor immunogenicity of tumors due to lack of costimulators and/or inability to stimulate class II MHC–restricted helper T cells, downregulation of MHC molecules, induction of tolerance to tumor antigens, loss of expression of immunogenic proteins due to mutations, modulation of tumor antigens by anti-tumor antibodies, and immunosuppression of the host. Treatment of tumors by immunologic approaches has not yet succeeded on a large scale, but new techniques are being tested. Strategies to enhance T cell–mediated anti-tumor immunity, adoptive cellular immunotherapy, and cytokine treatment all continue to be investigated.

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EXHIBIT B

[CANCER RESEARCH 57, 4465–4468, October 15, 1997]

Advances in Brief

Induction of Antitumor Cytotoxic T Lymphocytes with a MAGE-3-encoded Synthetic Peptide Presented by Human Leukocytes Antigen-A24[1]

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Abstract

For the development of immunotherapy using MAGE peptides, the identification of additional tumor antigens is required. Because HLA-A24 is the most common allele in Japanese and is also frequently present in Caucasians, MAGE-3-encoded synthetic peptides with binding affinity for HLA-A24 were thus tested for the induction of specific CTLs from the peripheral blood mononuclear cells (PBMCs) of HLA-A24 healthy donors using a simplified method. By using a peptide with a sequence of IMPKAGLLI (amino acid position in MAGE-3 195-203), the CTL responses could thus be induced from unseparated PBMCs by stimulation with freshly isolated, peptide-pulsed PBMCs as antigen-presenting cells (APCs) and by also using interleukin 7 and keyhole limpet hemocyanin for a primary culture. The induced CTLs could lyse HLA-A24 carcinoma cells expressing MAGE-3, as well as the peptide-pulsed target cells, in an HLA class-I restricted manner. The identification of the MAGE-3/HLA-A24 peptide, IMPKAGLLI, may thus potentially offer the opportunities to design peptide-based immunotherapeutic approaches that might prove to be effective in treating patients with MAGE-3-positive malignant tumors.

Introduction

The antigenic peptides derived from the tumor antigen gene, *MAGE*, thus appear to serve as a target for CTLs in the context of HLA class I molecules, and some of these peptides have already been identified. Because the *MAGE* genes have been shown to be expressed in various tumors but not in normal tissue except for the testis (1), the tumor antigens thus appear to be potential targets for tumor-specific immunotherapy using antigenic peptides. Therefore, immunization using these MAGE peptides are presently under investigation (2).

Although several antigenic peptides encoded by *MAGE* genes have already been described (3–7), the identification of additional tumor antigens is still required for the development of this type of immunotherapy. For the identification of potential peptides, peptides capable of binding with a high affinity to target HLA alleles can thus be defined based on the screening of sequences for the presence of MHC binding motifs (8, 9). In addition, the immunogenicity of these potential peptides could also be tested by primary *in vitro* CTL induction using PBMCs[3] obtained from normal HLA-typed individuals (10). Using this approach, the antigenic peptides derived from MAGE-3 presented by HLA-A1 (10), -A2 (6), and -B44 (7) have thus been identified. Because the *MAGE-3* gene is expressed in a high proportion of melanoma as well as several other types of tumors (11–14), the MAGE-3 antigen presented by HLA-A24, which is the most common allele in Japanese (15) and is also frequently present in Caucasians (16), might, therefore, have a wide applicability for immunotherapy in the treatment of melanoma and other tumors.

We recently developed a simplified method to efficiently induce MAGE-specific CTLs from the PBMCs of a healthy donor by *in vitro* stimulation with MAGE peptide (17). In the present study, we investigated the induction of MAGE-3-specific CTLs from the PBMCs of HLA-A24 healthy donors by *in vitro* stimulation with a MAGE-3 synthetic peptide binding with a high affinity to the HLA-A24 molecule by using our newly developed method to identify the potential peptide derived from a *MAGE-3* gene presented by HLA-A24.

Materials and Methods

Cell Lines. The TISI cells, a human B-lymphoblastoid cell line showing HLA-A24, were supplied by the Takara Shuzo Co., Ltd. (Otsu, Shiga, Japan). The EBV-transformed B lymphoblastoid cells, MH1, were established in our laboratory using PBMCs from a blood donor in this study using a technique described previously. The colon carcinoma cell line WiDr, the breast cancer cell line MRKnu1, the gastric carcinoma cell line Kato-III, and K562 were all supplied by the Japanese Cancer Research Bank (Tokyo, Japan). The esophageal cancer cell line TE11 was provided by the Tohoku University Cell Bank (Sendai, Japan). These cell lines were maintained in RPMI 1640 containing 10% fetal bovine serum and antibiotics.

Synthetic Peptides. The peptides used for the MHC binding assays were purchased from Chiron/Mimotopes (Clayton, Victoria, Australia). Peptides used as radioactive probes were synthesized in-house, high performance liquid chromatography purified, and radioiodinated as described previously (9).

MHC Binding Assay. The peptide binding assay specific for the class I MHC molecule was also described previously (9). In brief, the assay is based on the inhibition of binding of radiolabeled standard peptides to detergent solubilized MHC molecules. The sequence of the standard peptide used for the MHC binding study for HLA-A24 was AYIDNYNKF. The standard peptide was radioiodinated using ^{125}I by the chloramine T method. HLA-A concentrations, yielding approximately 15% of bound peptide, was used in the inhibition assay. Various doses of the test peptides (10 μM to 1 nM) were incubated together with 5 nM of radiolabeled standard peptide and HLA-A24 molecules for 2 days at room temperature in the presence of a cocktail of protease inhibitors and 1 μM β_2-microglobulin. At the end of the incubation period, the percentage of MHC-bound radioactivity was determined by gel filtration.

CTL Induction Using Synthetic MAGE-3 Peptides. The CTL induction *in vitro* was performed according to the procedure as described previously (17). In brief, the PBMCs of a healthy donors (HLA-A24) were collected by centrifugation on a Ficoll-Paque density gradient. The PBMCs were prepulsed by purified peptides at a final concentration of 20 μg/ml for 3 h at 37°C. The cells were cultured in an RPMI 1640 supplemented with 5% heat-inactivated AB serum, 100 units/ml penicillin, 100 mg/ml streptomycin sulfate (Life Technologies, Inc., Grand Island, NY), and 50 μM 2-mercaptoethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), with the addition of keyhole limpet hemocyanin (5 μg/ml, Calbiochem-Novabiochem Co., San Diego, CA) and IL-7 (25 ng/ml; Upstate Biotechnology, Inc., Lake Placid, NY). On day 3, recombinant IL-2 was then added to the culture at 30 IU/ml. The responder

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[3] The abbreviations used are: PBMC, peripheral blood mononuclear cell; IL, interleukin.

cells were restimulated every 7 days with freshly isolated autologous PBMCs that had been prepulsed with peptide and treated with mytomicin C (Kyowa Hakko Co., Ltd., Osaka, Japan). The cultures were fed with a fresh medium containing IL-2 1 day after every restimulation. The CTL activity was assessed on day 28.

Phenotypic Analysis. Flow cytometry of the CTLs was performed using a FACScan. The cells were stained with murine anti-human monoclonal antibodies against CD3, CD4, and CD8 (Becton Dickinson, San Jose, CA). Isotype-matched murine antibodies (Becton Dickinson) served as negative controls.

Cytotoxicity Assay. The target cells were labeled with 100 μCi of sodium ^{51}chromate (^{51}Cr) for 1 h at 37°C, and the labeled cells were then washed and resuspended. The peptide-pulsed targets, TISI cells, were prepared by incubating the cells with the peptides (20 μg/ml) overnight at 37°C and then labeling them with ^{51}Cr. The effector cells were placed in each well of round-bottomed microtiter plates. The labeled target cells were then added to the well at a concentration of 5×10^3 cells/well to produce a total volume of 0.2 ml. After a 4-h incubation period, the release of the ^{51}Cr label was then measured by collecting the supernatant, followed by quantitation in an automated gamma counter. The percentage of specific cytotoxicity was calculated as the percentage of specific ^{51}Cr release: [(experimental ^{51}Cr release − control ^{51}Cr release)/(maximum ^{51}Cr release − control ^{51}Cr release)] × 100. To eliminate any nonspecific lysis due to natural killer-like effectors, the cytolytic activity was tested in the presence of a 30-fold excess of unlabeled K562 cells.

Inhibition of Cytotoxicity with Monoclonal Antibodies. Appropriate target cells were incubated with monoclonal antibodies at a final concentration of 1:20 for 1 h at 4°C prior to the assay for cytotoxicity. The monoclonal antibody used was anti-HLA class I antibody (Immunotech, Marseille, France).

Reverse Transcription-PCR Analysis of MAGE-3 Expression. Total RNA was isolated from the tumor cell lines using the acid guanidinium thiocynate-phenol-chloroform extraction procedure (18). cDNA was synthesized by reverse transcription from 2.5 μg of total RNA as described previously. MAGE-3 cDNA was detected by PCR amplification using oligonucleotide primers specific for the different exons of the *MAGE-3* gene (13, 14). The primer sequences were 5′-TGGAGGACCAGAGGCCCCC-3′ (AB-5) and 5′-GGACGATTATCAGGAGGCCTGC-3′ (BLE-5). PCR was performed for 33 cycles (30 s at 94°C, 40 s at 72°C). The PCR product was size-fractioned on 1% agarose gel. To ensure that the RNA had not degraded, a PCR assay with primers specific for the gene glyceraldehyde-3-phosphate dehydrogenase cDNA was thus carried out in each case. The primers used for the amplification of glyceraldehyde-3-phosphate dehydrogenase were 5′-GTCAACGGATTTGGTCGTATT-3′ and 5′-AGTCTTCTGGGTGGCAGTGAT-3′ (19).

Results

Expression of MAGE-3 mRNA. We analyzed the expression of MAGE-3 mRNA in all cell lines used in this study (Fig. 1). The *MAGE-3* gene was expressed in WiDr (colorectal cancer cell line, HLA-A24$^+$), MRKnu1 (breast cancer cell line, HLA-A24$^+$), TE11 (esophageal cancer cell line, HLA-A24$^+$), and Kato-III (gastric carcinoma cell line, HLA-A24$^-$). However, the expression of the *MAGE-3* gene was not observed in MH1 (EBV-transformed autologous B cell, HLA-A24$^+$) and TISI (human B-lymphoblastoid cell line, HLA-A24$^+$).

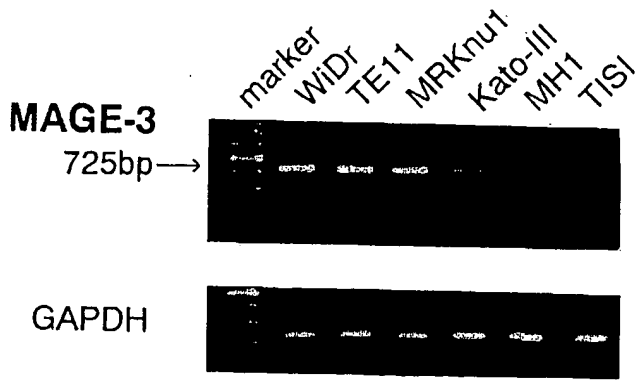

Fig. 1. Expression of the *MAGE-3* gene in cells, detected by RT-PCR and agarose gel electrophoresis. PCR amplification was performed with specific oligonucleotides.

Table 1 *HLA-A24 binding of motif containing nonapeptides derived from the sequence of MAGE-3*

Sequence	Position[a]	Binding[b] (IC_{50}, nM)
NWQYFFPVI	142	11
IMPKAGLLI	195	14
VAELVHFLL	113	149
NYPLWSQSY	76	363
IFSKASSSL	150	363

[a] Refers to the residue number of the first position of the peptide in relation to the sequence of the entire gene product.
[b] Concentration of the peptide necessary to inhibit 50% binding of the radiolabeled test peptide to purified HLA-A24 molecules.

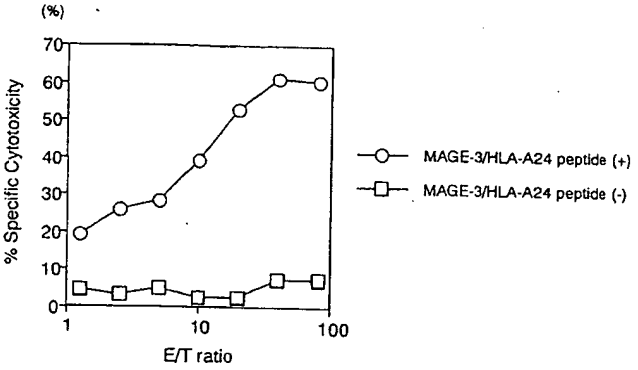

Fig. 2. The cytotoxic activity of the effector cells induced by stimulation with MAGE-3/HLA-A24 peptide IMPLAGLLI against TISI cells pulsed with the peptide. The effector cells were obtained by stimulating PBMCs for 28 days with mitomycin C-treated autologous PBMCs pulsed with the peptide. TISI cells were pulsed either with or without 20 μg/ml of this peptide overnight and then were labeled with ^{51}Cr. The cytotoxic activity against the TISI cells pulsed either with or without the peptide was assessed at various E:T ratios.

Identification of Peptides from MAGE-3 That Bind to HLA-A24. Initially, the known sequences of MAGE-3 were screened for peptides containing the anchor motif for HLA-A24 (9). Five peptides of nine residues were found to contain the binding motif for HLA-A24. These peptides were synthesized and tested for binding to purified HLA-A24 molecules. The results from the binding assay showed that two peptides bound to HLA-A24 with high affinity (<50 nM required to achieve 50% inhibition), and three peptides were intermediate HLA-A24 binders (50–500 nM required for 50% inhibition; Table 1).

Induction of CTLs using MAGE-3-derived Peptides. Using the simplified method for CTL induction, we stimulated PBMCs from at least two normal volunteers with the synthetic peptides. Of five peptides studied, a high MHC binder (IMPKAGLLI) was able to elicit CTLs. After 28 days of culture, the induced effector cells exhibited more than 60% cytotoxicity against the peptide-pulsed TISI cells, compared with the less than 10% cytolysis observed against TISI cells alone, at the E:T ratio of 80:1 (Fig. 2). Furthermore, the induced cells were also able to lyse WiDr (MAGE-3$^+$, HLA-A24$^+$), TE11 (MAGE-3$^+$, HLA-A24$^+$) cells, and MRKnu1 (MAGE-3$^+$, HLA-A24$^+$). However, the cytolysis was minimal against Kato-III (MAGE-3$^+$, HLA-A24$^-$) and MH1 (MAGE-3$^-$, HLA-A24$^+$) cells (Fig. 3). No anti-peptide or antitumor reactivities were detected in the case of the remaining four peptides, except for the peptide VAELVHFLL, which is presently under investigation.

Characterization of the Cytotoxic Effectors. A flow cytometric analysis was performed before and after the culture in the case of the

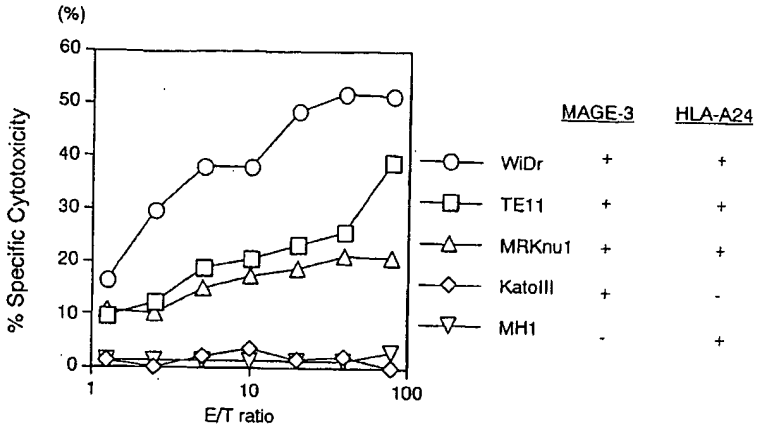

Fig. 3. Lysis of various tumor cell lines by the effector cells induced by using the peptide IMPKAGLLI. The cytotoxic activity of the effector cells was assessed against WiDr (colorectal carcinoma cell line, HLA-A24$^+$, MAGE-3$^+$), TE11 (esophageal carcinoma cell line, HLA-A24$^+$, MAGE-3$^+$), MRKnu1 (breast carcinoma cell line, HLA-A24$^+$, MAGE-3$^+$), Kato-III (gastric carcinoma cells, HLA-A24$^-$, MAGE-3$^+$), and MH1 (EBV transformed autologous B cell line, HLA-A24$^+$, MAGE-3$^-$), at various E:T ratios.

peptide IMPKAGLLI (Fig. 4). Following the culture, the number of CD3$^+$ gradually increased. The percentage of CD4$^+$ cells continued to increase until day 21 and thereafter decreased, whereas the CD8$^+$ cells increased after day 21. On day 28, 98% of the cell population was CD3$^+$, and 80% of these cells were positive for CD8.

Inhibition of the Recognition of Effectors by Monoclonal Antibody. To determine whether the induced effector cells using the peptide IMPKAGLLI recognized the MAGE-3 expressing targets in a HLA-restricted manner, the monoclonal antibody generated against HLA class I molecules was used to block the recognition by effectors. The cytotoxic activity of the effector cells against WiDr, TE11, and MRKnu1 was thus significantly eliminated by the anti-HLA-class-I antibody (Fig. 5). These results, therefore, suggest that the induced effectors lysed the target cells expressing MAGE-3 in an HLA class I-restricted manner.

Discussion

The antigenic peptides derived from the *MAGE-3* gene that can be recognized in the context of HLA class-I molecules have been investigated. The MAGE-3-encoded peptide presented by HLA-A1 has also been identified by using tumor-specific HLA-A1-restricted CTL clones derived from autologous PBMCs stimulated with irradiated tumor cells of a melanoma patient (4). On the other hand, the same MAGE-3 peptide was thus reported to be independently identified by the *in vitro* primary induction of peptide-specific CTLs that lysed the HLA-A1 tumor expressing MAGE-3 from the PBMCs of healthy donors using a high-affinity HLA-A1 binding synthetic peptide (10). Using the latter approach, CTLs that recognized HLA-A2 tumor cells expressing MAGE-3 were thus obtained from a MAGE-3 peptide that bound to HLA-A2 (6). Furthermore, the HLA-B44-restricted MAGE-3 peptide has been reported previously to be identified using similar procedures (7). In the present study, we induced specific CTLs that lysed HLA-A24 tumor cells expressing MAGE-3 in an HLA class I-restricted manner by the *in vitro* stimulation of PBMCs in an HLA-A24 healthy donor with an HLA-A24 binding synthetic peptide derived from MAGE-3 IMPKAGLLI, by using our simplified method reported previously (17).

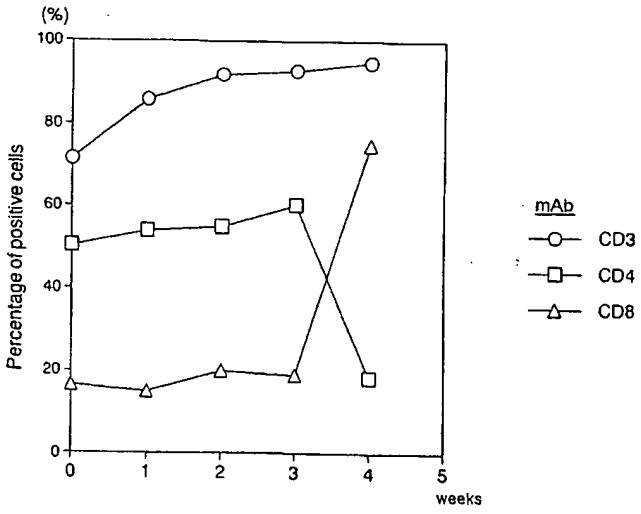

Fig. 4. Phenotype of the effector cells stimulated with MAGE-3/HLA-A24 peptide IMPKAGLLI. Effector cells generated from a healthy donor were stimulated with the MAGE-3 peptide, and these cells were stained with anti-CD3, anti-CD4, and anti-CD8 monoclonal antibody.

For the *in vitro* induction of peptide-specific CTLs from PBMCs, complicated manipulations are required (6, 7, 10). These procedures include the sorting of CD8$^+$ T cells; the usage of several cytokines such as IL-2, IL-4, IFN-γ, IL-6, IL-7, and IL-12; and the generation of lymphoblastoid cells or dendritic cells such as antigen-presenting cells. However, we recently developed a simplified method to generate peptide-specific CTLs from the PBMCs of healthy donors by stimulation with MAGE peptide, in which the CTL responses were induced from unseparated PBMCs, by using IL-7 and keyhole limpet hemocyanin for primary culture and then freshly isolated PBMCs for every stimulation as antigen-presenting cells (17). Using this simple method, a MAGE-3-encoded peptide binding to HLA-A24 was thus able to induce HLA-A24-restricted, MAGE-3-specific CTLs from the PBMCs of a healthy donor in this study.

In a previous experiment on the generation of MAGE-3/HLA-A2 peptide-specific CTLs, the induced CTLs could lyse the peptide-pulsed target cells, whereas a significant level of lysis by the induced CTLs was observed only on the HLA-A2 tumor cells expressing MAGE-3 that had been pretreated with IFN-γ and TNF-α to induce a higher expression of HLA class I molecule (6), even though the peptide-specific CTLs induced by using MAGE-1/HLA-A1 or MAGE-3/HLA-B44 peptide were able to kill the target cells that endogenously presented the MAGE-3 epitope (7, 10). Our results showed that significant levels of lysis could be detected against HLA-A24 carcinoma cells expressing MAGE-3, without pretreatment with these cytokines, as well as peptide-pulsed target cells, when the CTLs were induced by stimulation with a MAGE-3/HLA-A24 peptide.

Because the *MAGE* genes were not expressed in normal tissues with the exception of the testis, the antigens encoded by these genes

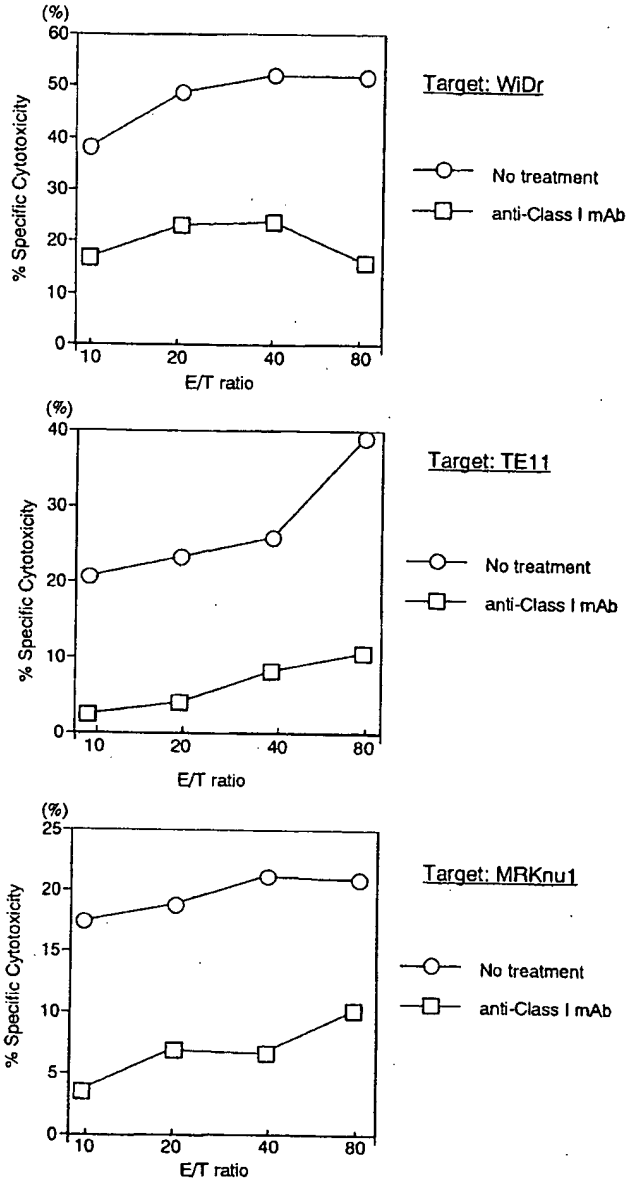

Fig. 5. Inhibition of the recognition of the effector cells by the anti-HLA class I antibody. The target cells, WiDr, TE11, and MRKnu1, were incubated with monoclonal anti-HLA class I antibody at a final concentration of 1:20 for 1 h at 4°C. The cytotoxic activity of the effector cells was assessed against the target cells pretreated either with or without the antibody at various E:T ratios.

may thus be potentially useful targets for tumor-specific immunotherapy. Marchand *et al.* (2) demonstrated that three melanoma patients of 12 HLA-A1 patients expressing MAGE-3 displayed a very significant degree of tumor regression after treatment with the MAGE-3-encoded peptide presented by HLA-A1 alone. These results suggest that peptide immunization may, therefore, be potentially effective in a significant proportion of melanoma patients.

The antigenic peptides encoded by the *MAGE-3* gene were shown to be presented by either the HLA-A1, A2, or B44 molecule (4, 6, 7, 10). The proportion of HLA-A1 or B44 individuals is less than 1 or 7% in Japanese but is 26 or 24% in Caucasians, whereas 44% of Japanese and 49% of Caucasians express the *HLA-A2* allele. However, HLA-A24 is present in 61% of Japanese (15) but only in 10% of Caucasians (16). It would, therefore, be possible to immunize many more patients with malignant tumors, especially Japanese patients, against the antigens encoded by the *MAGE-3* gene, if the MAGE-3/HLA-A24 peptide, which was identified in this study, can be used for immunization against HLA-A24 patients bearing MAGE-3-positive malignant tumors. Furthermore, the identification of this peptide will also permit the immunization of patients against several antigens concurrently expressed by their tumors, which may thus also improve the effectiveness of the antitumor response.

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Immunotherapy of Bladder Cancer Using Autologous Dendritic Cells Pulsed with Human Lymphocyte Antigen-A24-specific MAGE-3 Peptide

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ABSTRACT

Recent investigations have demonstrated the efficacy of autologous dendritic cells (DCs) pulsed with tumor antigens to generate tumor-specific CTLs against cancer cells. Melanoma antigens (MAGE) are a family of tumor-specific antigens shown to be expressed in various tumors, including bladder cancers and melanoma, but not in normal tissues except for the testis. Because invasive bladder cancers are frequently reported to express MAGE, we explored the possibility of establishing a new immunotherapeutic modality against advanced bladder cancer using autologous DCs pulsed with one of the MAGE-3 epitope peptides (IMPKAGLLI), which is synthesized to bind specifically to HLA-A24. A MAGE-3-expressing bladder cancer cell line, FY, was newly established from a lymph node metastasis of bladder cancer in a HLA-A24+ patient. The FY cell-specific CTL response was significantly higher when CTL was induced by autologous DCs pulsed with IMPKAGLLI than by FY cells alone or by nonpulsed DCs *in vitro*. A total of four HLA-A24+ patients with advanced MAGE-3+ bladder cancers were treated with s.c. injections of autologous DCs pulsed with IMPKAGLLI every 2 weeks for a minimum of 6 and a maximum of 18 times. Three of four patients showed significant reductions in the size of lymph node metastases and/or liver metastasis. No significant untoward side effects were noted in these patients. This study indicated that, at sometime in the future, tumor-specific DC-based cancer immunotherapy may be useful as an additional treatment modality against advanced bladder cancer.

INTRODUCTION

Several tumor antigens recognized by CTLs have been identified in multiple types of solid tumors (1). Many of these antigens are derived from tissue-specific differentiation antigens (2, 3), from oncogenes (4, 5), or from a set of antigens expressed preferentially in tumors (6–9). Because of recent progress in understanding tumor-specific antigens that can potentially stimulate CTL and Th[2] -cell responses, efforts to develop peptide- and cell-based tumor vaccines are increasing (10, 11). Melanoma antigens such as the MAGE family are now well known as tumor-rejection antigens recognized by CTLs in a HLA-restricted manner (12–14). MAGE is reported to be expressed in a broad range of cancers including melanoma (6), esophagus (15), breast (16), lung (17), and bladder (18) but not in normal tissues except for the testis, which does not express HLA class I molecules (19, 20). Therefore, these antigens are considered to be attractive targets for anticancer immunotherapy. DCs are the most potent professional antigen-presenting cells for inducing anticancer immunity both *in vitro* and *in vivo*. Several clinical studies already have demonstrated the potential efficacy of active immunotherapy using DCs loaded with various tumor-specific antigens *in vitro* (21–24).

Bladder cancers constitute a broad spectrum of malignancies at clinical presentation. Various chemotherapies with multiple anticancer reagents have only limited efficacy against highly advanced disease and have considerable systemic side effects (25). On the other hand, superficial bladder cancer, especially carcinoma *in situ* disease, is well known to respond to immunotherapy such as intravesical treatment by *Bacillus Calmette-Guérin*, and thus tumor-specific immunotherapy has been suggested as a potentially useful strategy against bladder cancer (26). In the present study, we were able to establish a MAGE-3+ bladder cancer cell line from a HLA-A24+ patient with advanced bladder cancer. Recently, Tanaka *et al.* (14) reported the successful induction of antitumor CTLs with a MAGE-3-encoded synthetic peptide presented by HLA-A24. In this previous report, five peptides of nine amino acids were found to contain the binding motif for HLA-A24 in the known sequence of MAGE-3. These peptides were synthesized and tested for their binding ability to purified HLA-A24 molecules. Of the five peptides studied, one high MHC binder (IMPKAGLLI) was found to be capable of eliciting CTLs. Using this particular model, we explored the possibility of inducing a

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[2] The abbreviations used are: Th, T-helper; DC, dendritic cell; MAGE, melanoma antigens, RT-PCR, reverse transcriptase-PCR; HLA, human lymphocytic antigen; PBMC, peripheral blood mononuclear cell; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; mAb, monoclonal antibody.

significant MAGE-specific CTL response by the *in vitro* stimulation of PBMCs with autologous DCs pulsed with the MAGE-3 synthetic epitope peptide, IMPKAGLLI, which binds to HLA-A24 molecules with a very high affinity. On the basis of this achievement, a pilot clinical trial of DC vaccination targeting MAGE-3 has been conducted in selected HLA-A24+ patients with advanced bladder cancer proven to be MAGE-3+.

MATERIALS AND METHODS

Cell Lines. Five established human bladder cancer cell lines, KU-1, KU-7 (27), KU-19-19 (28), T24 (29), and a newly established FY cell line were evaluated. The FY cell line was established from the lymph node metastasis of a bladder cancer in a 76-year-old HLA-A24+ Japanese female patient. In March, 1996, the patient underwent a radical cystectomy for muscle invasive bladder cancer. The histological evaluation revealed a TCC = SCC > AC, grade 3, pT3, nonpapillary invasive tumor. The HLA typing of this patient was A24 (9), B7, B52 (5), and Cw7. Metastases to the right inguinal and para-aortic lymph nodes were apparent in August, 1996. A primary culture was established from the specimens taken from the right inguinal lymph node metastasis. K562 cell line was kindly supplied from the Japanese Cancer Research Bank (Tokyo, Japan). The cell lines were well maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and antibiotics.

Clinical Tumor Samples. Specimens of the 28 bladder cancers and 8 upper tract urothelial cancers as well as normal bladder mucosa samples were obtained from the patients at the time of surgery performed at Keio University Hospital. A routine histopathological evaluation was conducted, and a portion of each sample was immediately frozen in liquid nitrogen and stored at −80°C for later RNA extraction.

Analysis of mRNA Expression. Total RNA was isolated from each of the samples using the acid guanidinium thiocyanate-phenol chloroform extraction procedure (30). As described by De Smet *et al.* (31), cDNA synthesis from 2.5 μg of total RNA was accomplished by extension with oligo$(dT)_{15}$ in a 20-μl reaction volume. Each of the MAGE cDNA samples was detected by PCR amplification by 33 cycles (30 s at 94°C and 40 s at 72°C) using oligonucleotide primers specific for the different exons of each *MAGE* gene. The sequences were as follows: (*a*)5′-CGGCCGAAGGAACCTGACCCAG-3′ (CHO-14) and 5′-GCTGGAACCCTCACTGGGTTGCC-3′ (CHO-12) for the *MAGE-1* gene (16); (*b*) 5′-AAGTAGGACCCGAGGCACTG-3′ (CDS-9) and 5′-GAAGAGGAAGAAGCGGTCTG-3′ (CDS-7) for the *MAGE-2* gene (31); and (*c*) 5′-TGGAGGACCAGAGGCCCCC-3′ (AB-1197) and 5′-GGACGATTATCAGGAGGCCTGC-3′ (BLE-5) for the *MAGE-3* gene (17). To ensure that the RNA had not degraded, a PCR assay for the *glyceraldehyde-3-phosphate dehydrogenase* gene also was carried out; the sense primer was 5′-GTCAACGGATTTGGTCGTATT-3′ and the antisense primer was 5′-AGTCTTCTGGGTGGCAGTGAT-3′ (32). The PCR product was size-fractionated on 1% agarose gel.

Statistical evaluations regarding the relationship between the *MAGE* gene expression and the disease stages were analyzed by the Mann-Whitney nonparametric *U* test.

Generation of Dendritic Cells. PBMCs were isolated from the heparinized venous blood of the patient from whom the FY cell line was established, by Ficoll-Hypaque (Lymphoprep; Nycomed Labs, Oslo, Norway) gradient centrifugation at 580 × *g* for 20 min and then washed with PBS three times. The adherent monocytes were cultured in RPMI 1640 supplemented with 5% human AB serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 μg/ml gentamicin, 2,000 units/ml recombinant IL-4 (R & D Systems, Inc., Minneapolis, MN), and 1,000 units/ml recombinant GM-CSF (R & D Systems, Inc.) for 7 days at 37°C. At the time of CTL induction *in vitro*, the DCs were pulsed with 10 μg/ml of the MAGE-3 epitope peptide for 4 h at 37°C in a serum-free medium, washed extensively, and then added to the bulk cultures of the PBMCs.

Phenotyping of DCs. The expressions of the cell surface antigens, MHC classes I and II, CD3, CD14, CD83, and CD86, were analyzed by flow cytometry using mAbs before and after the generation of DCs to evaluate the populations of the DCs generated from the PBMCs. The mAbs used were G46–2.6 (IgG1, anti-HLA-A, B, C), L243 (IgG2a, anti-HLA-DR; BD Biosciences, San Jose, CA), SK7 (IgG1, anti-CD3), SK3 (IgG1, anti-CD4), SK1 (IgG1, anti-CD8), MϕP9 (IgG2b, anti-CD14; Becton Dickinson, San Jose, CA), HB-15e (IgG1, anti-CD83), and BU63 (IgG1, anti-CD86; Ancell, Bayport, MN). All of the data were analyzed using the CellQuest software (Becton Dickinson).

Synthetic Epitope Peptide. A nine amino acids-peptide with a sequence of IMPKAGLLI (amino acid position in MAGE-3, 195–203) was synthesized and provided by Takara Shuzo, Shiga, Japan. This MAGE-3 synthetic epitope peptide was evaluated previously and identified to bind to HLA-A24 molecules with a very high affinity (14).

Induction of CTL Responses. The PBMCs were collected from patient F. Y. and from HLA-A24+ healthy donors by the centrifugation of blood samples on a Ficoll-Hypaque density gradient as described above and cultured in RPMI 1640 containing 5% heat-inactivated human AB serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (Life Technologies, Inc.). As a stimulator, autologous DCs pulsed with MAGE-3 epitope peptide (PBMC:DC, 10:1) were added to the media every 7 days. Irradiated FY cells or nonpulsed autologous DCs were used as a control stimulator (PBMC:FY cells, 10:1). Recombinant IL-2 (R & D Systems, Inc.) was added to the media at a concentration of 300 units/ml every 3 days. The whole culture media was replaced on days 9 and 16. After 21 days of incubation, the cells were harvested, and the CTL activity was assessed. A flow cytometric analysis of the cell-surface antigens of the effector cells was performed. The cells were stained with mouse antihuman mAbs against CD3, CD4, and CD8 (Becton Dickinson). Isotype-matched mouse antibodies (Becton Dickinson) served as a negative control.

Cytotoxicity Assay. The target cells were labeled with ^{51}Cr by incubating with 100 μCi of $Na^{51}CrO_4$ (New England Nuclear, Boston, MA) for 1 h at 37°C, washed four times, and plated onto round-bottomed, 96-well microtiter plates at a concentration of 5×10^3 cells/0.1 ml/well. Effector cells were then added at a concentration of 2.5×10^4 cells/0.1

Table 1 The expression of MAGE genes according to pathological stages in bladder and upper tract urothelial cancers[a,b]

Pathological stage	No. of tumors	No. of MAGE + tumors			
		MAGE-1	MAGE-2	MAGE-3	Any of three
Bladder cancer					
<pT1	17	1	1	1	1 (6%)
pT2≤	11	4	3	6	8 (73%)[c]
Total	28	5	4	7	9 (32%)
Upper tract urothelial cancer					
<pT1	4	2	1	1	2 (50%)
pT2≤	4	3	4	4	4 (100%)
Total	8	5	5	5	6 (75%)

[a] Clinical tissue samples were obtained from the patients at the time of surgery performed at Keio University Hospital.
[b] Expression of *MAGE* genes was detected by RT-PCR amplification of total RNA isolated from each sample using specific oligonucleotide primers.
[c] Statistical significance between stages <pT1 and pT2≤ was analyzed by Mann-Whitney nonparametric U test. $P = 0.0003$.

Table 2 The expression of MAGE genes and HLA typing in established bladder cancer cell lines[a,b]

Cell line	Pathology	MAGE-1	MAGE-2	MAGE-3	HLA-A2	HLA-A24
KU-1	TCC, G2	−	−	−	−	−
KU-7	TCC, G1	++	±	+++	+	−
KU-19-19	TCC, G3	−	+	+++	+	−
T24	TCC, G2	−	+	±	+	+
FY	TCC, G3	++	+++	+++	−	+

[a] Expression of *MAGE* genes was detected by RT-PCR amplification of total RNA isolated from cell lysates of each cell line using specific oligonucleotide primers.
[b] The relative levels of gene expression were determined as follows: the strongest positive band was defined as 100% and graded "−" for no expression; ±, <2% positive; +, 2–25% positive; ++, 26–50% positive; +++, >50% positive.

ml/well (effector:target ratio 5:1). To eliminate nonspecific lysis, the cytotoxic activity was tested in the presence of a 30-fold excess of unlabeled K562 cells. After incubation for 4 h, the release of ^{51}Cr in the supernatant was measured by an automated gamma counter. The percentage of specific ^{51}Cr release was calculated by the following formula: 100 × ([release by CTL] − [spontaneous release])/([maximum release] − [spontaneous release]).

Spontaneous release was generally 15–20% of the maximum release in our experiments.

Statistical evaluations between the relationship of the CTL activity and the methods of stimulation of effector cells were analyzed using Student's t test.

Inhibition of Cytotoxicity by Blocking mAbs. The target cells were incubated with 10 μg/ml of anti-HLA class I mAbs (Immunotech, Marseille, France) for 1 h at 4°C before the CTL assay.

Patient Eligibility and Clinical Protocol. The protocol was reviewed and approved by the Keio University Hospital Institutional Protocol Review Committee, and all of the patients provided signed informed consent that fulfilled the committee's guidelines. A total of four HLA-A24+ patients with advanced bladder cancers were eligible and enrolled in our pilot clinical study. The diseases of these patients were progressive despite the fact that previously they had had surgery, chemotherapy, and radiotherapy. The expression of the *MAGE-3* gene in all four cancers was proved by RT-PCR in at least one excised tumor. None of these patients received other treatments within the 4 weeks preceding the initial DC vaccination. The autologous DCs were prepared from PBMCs obtained from leukapheresis and were cryopreserved at −80°C. While preparing the DCs, samples were collected from each step and tested for sterility, including bacterial, *Mycoplasma,* and endotoxin contamination. The DCs were pulsed with the MAGE-3-encoded HLA-A24 binding epitope (IMPKAGLLI) just before each vaccination, and the patients received a s.c. injection of 1×10^7 to 1×10^8 autologous DCs at each vaccination. The treatments were conducted once every 2 weeks for a minimum of 6 and a maximum of 18 times. No other concomitant treatments were provided during the protocol. The patients were carefully observed, and vital signs, clinical symptoms, and laboratory examinations were monitored and evaluated throughout the experiments.

RESULTS

Expression of *MAGE* Genes in Bladder and Upper Tract Urothelial Cancers. We analyzed the expression of MAGE mRNA in the clinical tissue samples obtained at the time of the surgery (Table 1). A total of 9 of 28 (32%) bladder cancers expressed any of the three *MAGE* genes. The *MAGE*+ bladder cancer was significantly more frequent in muscle invasive disease (pT2≤; 73%) than in superficial disease (<pT1; 6%). In upper tract urothelial cancers, all of the invasive disease (pT2≤) expressed at least one of the *MAGE* genes, whereas two of four (50%) of the superficial diseases (<pT1) did; however, the difference was not statis-

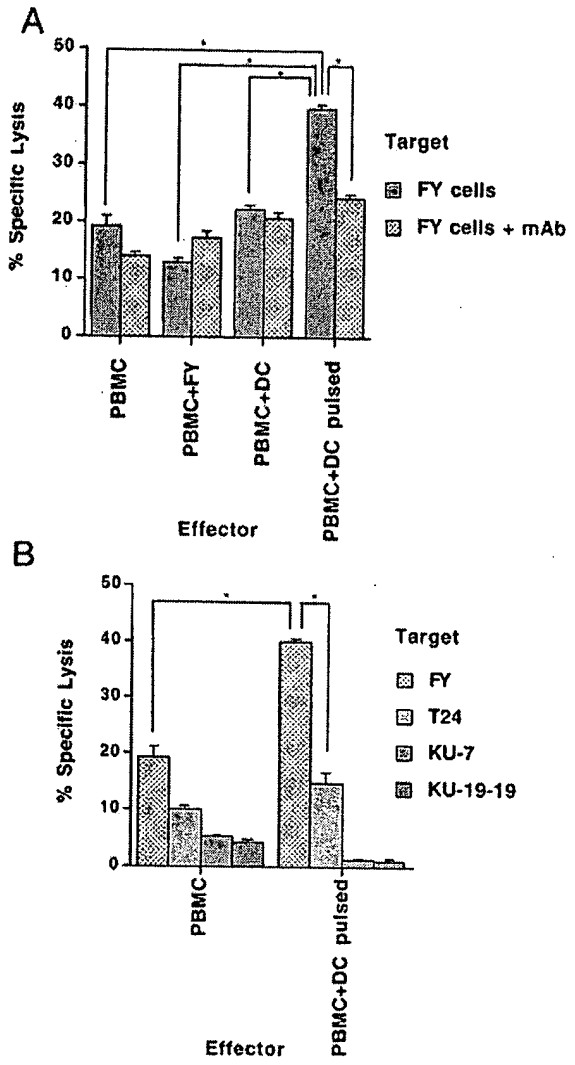

Fig. 1 *A*, cytotoxic activities of the effector cells from patient F. Y. against target FY cells and inhibition of CTL response by anti-HLA class I mAbs. PBMCs from patient F. Y. were collected and cultured as described in "Materials and Methods." Effectors were stimulated under four different conditions, as follows: (*a*) PBMCs only; (*b*) PBMCs + irradiated FY cells (10:1); (*c*) PBMCs + nonpulsed autologous DCs (10:1); and (*d*) PBMCs and DCs pulsed with MAGE-3-encoded HLA-A24-binding peptide (10:1). After 3 weeks of *in vitro* stimulation, a standard ^{51}Cr release CTL assay was carried out. Target FY cells (MAGE-3 and HLA-A24+) were either nontreated or preincubated with anti-HLA class I mAbs and then added at an effector:target ratio of 5:1 and incubated for 4 h. *, $P < 0.0001$ (Student's *t* test). *B*, cytotoxic activities of the effector cells from patient F. Y. against various target bladder cancer cells. Effector cells were prepared, and CTL assay was conducted as the same method described in Fig. 1*A*. Four different established bladder cancer cell lines were used as target cells. The characteristics of each cell line are shown in Table 2. *, $P < 0.0001$ (Student's *t* test).

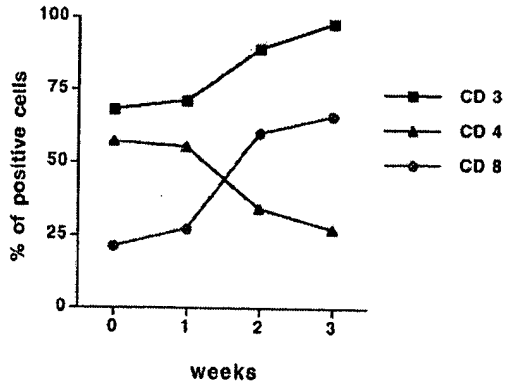

Fig. 2 Phenotype of the effector cells stimulated by autologous DCs pulsed with MAGE-3-encoded HLA-A24 binding peptide. Adherent PBMCs were collected from patient F. Y. and stimulated as described in "Materials and Methods." Flow cytometric analysis of effector cells was performed weekly during the induction of effector cells. Single representative data are shown.

tically significant. In the established human bladder cancer cell lines, all but KU-1 expressed *MAGE* genes (Table 2). None of the samples that we examined, which were obtained from renal cell carcinoma and adrenal tumors, expressed *MAGE* genes, whereas in five testicular cancers, all of the samples were positive for at least one of the *MAGE* genes.[3]

Dendritic Cell Preparation. Remarkable changes in the cell surface markers of PBMCs obtained from patient F. Y. were observed with the *in vitro* 7-day stimulation by IL-4 and GM-CSF. In the single representative evaluation of cell surface markers by flow cytometry, a markedly increased expression of MHC class II (from 44% population of the whole PBMCs before the generation of DCs to 99% population of the adherent monocytes after a 7-day stimulation by IL-4 and GM-CSF), CD83 (from 4% to 96%), and CD86 (from 12% to 99%) was obtained, whereas a remarkable decrease was observed in the expression of CD3 (T-cell marker; from 57% to 1%) and CD14 (macrophage marker; from 18% to 1%). These changes in the surface markers of the populations of PBMCs indicated the successful generation of DCs.

Induction of Autologous CTL Response against FY Cells by DCs Pulsed with HLA-A24-binding MAGE-3 Epitope Peptide. Compared with the CTL response stimulated only by FY cells or nonpulsed autologous DCs, the cytotoxicity was significantly higher in the stimulation with autologous DCs pulsed with HLA-A24-binding MAGE-3 epitope peptide. The cytotoxic effect of this CTL was significantly blocked by the pretreatment of target FY cells with anti-HLA class I mAbs (Fig. 1*A*). In addition, the induced CTL did not recognize both KU-7 and KU-19-19, MAGE-3+, and HLA-A24− bladder cancer cells, but in contrast, moderately recognized T24, HLA-A24+, and MAGE-3 weakly positive bladder cancer cells (Fig. 1*B*). PBMCs were also collected from a HLA-A24+ healthy donor, and the effector cells were prepared according to the same method. These HLA-matched, but heter-

[3] T. Nishiyama, unpublished data.

Table 3 Patients' profiles and responses to DC vaccination

	HLA-A24	MAGE-3	Sites of tumors	No. of DC vaccinations	Clinical response	Previous treatment	Survival[a]
F.Y. 75 yr F	+	+	Right inguinal LN[b] Para-aorta LN Right external iliac LN	6	Complete response[c]	Radical cystectomy Chemotherapy Radiation	2[d]
G.K. 65 yr M	+	+	Iliac LN Para-aorta LN Liver Brain[e]	18	Partial response of para-aorta LN and complete response of liver metastasis overall progression	Radical cystectomy Chemotherapy Radiation	8
N.H. 64 yr M	+	+	Bilateral inguinal LN Perineum	6	Partial response	TURBT Chemotherapy Radiation	5
N.T. 56 yr M	+	+	Local recurrence Pleural dissemination	6	Progressive disease	Radical cystectomy Chemotherapy Radiation	2

[a] Survival is defined as months to death after the DC vaccination.
[b] LN, lymph node.
[c] Determined by autopsy.
[d] Death attributable to perforation of small intestine.
[e] Solitary brain metastasis developed 6 months after the DC vaccination and was surgically removed.

ologous, CTLs were also shown to respond to target FY cells as well as to autologous CTLs.[4]

Characterization of the Cytotoxic Effectors. A flow cytometric analysis was performed during the induction of effector cells by DCs pulsed with the MAGE-3 epitope peptide, IMPKAGLLI (Fig. 2). The number of CD3+ cells gradually increased during the incubation. CD4+ cells decreased after 2 weeks, whereas the number of CD8+ cells kept increasing. CD3+ and CD8+ cells accounted for 98% and 66% of the effectors, respectively, after 3 weeks of induction.

Clinical Cases. A total of 4 HLA-A24+ patients who had metastatic MAGE-3+ bladder cancer with measurable lesions were treated in this pilot clinical trial (Table 3). These patients had already been treated intensively with surgery, chemotherapy, and radiotherapy. All of the bladder cancers resected from these patients were examined by RT-PCR and thus were proven to be MAGE-3+. None of the patients showed any unfavorable side effects throughout the DC vaccinations. A complete response of lymph node metastases was achieved in one patient, and a partial response was observed in two other patients. However, one patient died because of a progression of local recurrence and pleural dissemination. The first case (F. Y.) developed disseminated intravascular coagulation and died of sepsis attributable to a perforation of the small intestine 2 months after the DC vaccination; however, autopsy showed a complete remission of lymph node metastases, which were histologically evident before the DC vaccination. The second case (G. K.) showed the disappearance of a solitary liver metastasis and a >50% reduction in the size of the para-aortic lymph node metastasis evaluated by a computed tomography scan (Figs. 3 and 4). However, a solitary brain metastasis was apparent 6 months after the treatment, and was surgically removed. The third case (N. H.) showed a significant reduction in the size of inguinal lymph node metastases. A biopsy taken from the lymph node 3 months after the DC vaccination demonstrated significant necrotic changes in the lesions (Fig. 5).

DISCUSSION

MAGE genes are widely expressed in various cancers but not in normal tissues except for the testis, which makes *MAGE* potentially useful targets for tumor-specific immunotherapy. Patard *et al.* (18) reported that MAGE-1 and -3 were positive in 21% and 35% of tumors, respectively, in primary transitional cell carcinoma of the urinary bladder, and their expressions were more frequent in advanced stages of disease. In our current study, we first showed a high incidence of *MAGE* expression in bladder cancers and upper tract urothelial cancers. It was statistically significant and consistent with the findings of previous reports that the *MAGE* expression was observed more frequently as the tumor's pathological stages advanced. Hence, the presence of MAGE in advanced urothelial cancers is highly expected. As a result, MAGE is increasingly suggested to be a useful target for active immunotherapy against bladder cancer. In this study, to establish the possibility of a modality of immunotherapy for invasive bladder cancers, we hypothesized that using autologous DCs pulsed with HLA-specific MAGE epitope peptide could induce autologous CTLs against *MAGE*-expressing bladder cancers.

DCs are well known to be professional antigen-presenting cells for the induction of a T cell-mediated immune response (33). High expression levels of adhesion molecules and costimulators, such as B7, on the cell surface and intracellular vesicles critical for antigen presentation are believed to prime CD4+ and CD8+ T cells (34, 35). Another means by which DCs induce potent T cell responses is via the release of IL-12. Using murine T cell receptor transgenic CD4+ T cells, Macatonia *et al.* (36) showed that DCs induced the differentiation of naive T cells into IFN-γ producing Th cells by stimulating them with IL-12 production. In addition, DCs induce potent human antiviral CD8+

[4] T. Nishiyama, unpublished data.

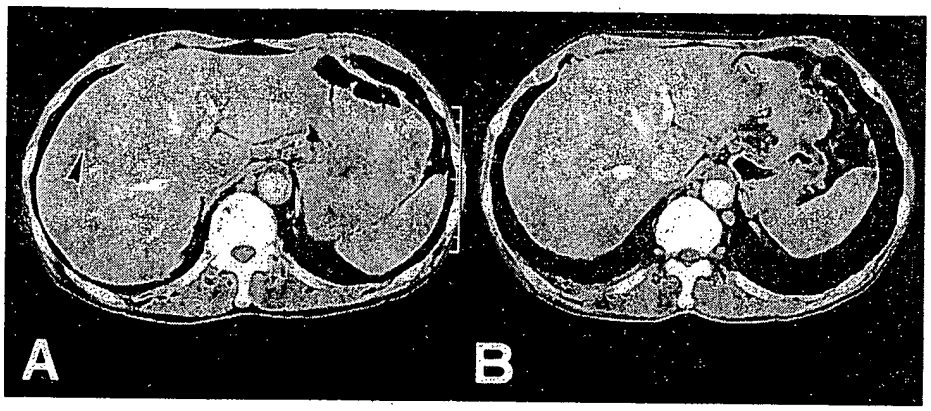

Fig. 3 Complete response of a metastatic lesion in liver by MAGE peptide-pulsed DC immunotherapy in a patient with advanced bladder cancer. CT scan shows a solitary liver metastatic lesion (*arrow* in Fig. 3*A*) completely disappeared after 18 × biweekly DC vaccination (*B*).

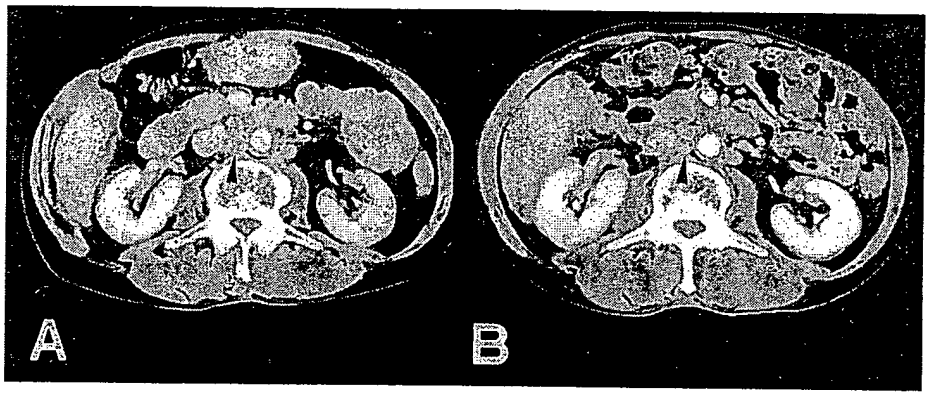

Fig. 4 Effectiveness of the MAGE peptide-pulsed DC immunotherapy against paraaortic lymph node metastasis of bladder cancer. A bulky paraaortic lymph node metastasis was observed by computed tomography scan before the MAGE peptide-pulsed DC vaccination (*A*; *arrow*) in the same patient as in Fig. 3. A >50% reduction in the size of metastasis was evident after the DC vaccination (*B*, *arrow*).

CTL responses without any need for CD4+ T cells or exogenous cytokines (37). As these previous reports have indicated, DCs and macrophages have the ability to induce CTL and Th cell responses *in vivo*. DCs are shown to process exogenous antigens conventionally for presentation on MHC class II molecules. Bachmann *et al.* (38) reported that a cloned DC line is able to present cell debris-associated exogenous viral proteins to MHC class I-restricted CTLs *in vitro*. Before using DCs in anticancer immunotherapy, the epitope peptide that is most essential to anticancer CTL induction must be identified to avoid the risk of developing autoimmune diseases (13).

The antigenic peptides encoded by the *MAGE-3* gene were shown to be presented by either HLA-A1 (39), -A2 (13), or -B44 (40) molecules (41), and Tanaka *et al.* (14) recently showed tumor-specific CTLs to be induced from healthy donors' PBMCs by stimulation with HLA-A24-binding MAGE-3-derived synthetic peptide, IMPKAGLLI. HLA-A24 is positive in ~61% of the Japanese population (42). As a result, it makes sense to develop current DC-based immunotherapy using this specific synthetic epitope peptide.

In our current experiment, we have shown the successful induction of the CTL response by stimulating PBMCs with autologous DCs pulsed with a HLA-A24-binding MAGE-3 epitope peptide. This CTL was considered to recognize MAGE-3+ tumor cells in a MHC class I-restricted manner because the cytotoxic effect of this CTL against FY cells was significantly blocked by the pretreatment of FY cells with anti-HLA class I mAbs. In addition, the induced CTL did not recognize either KU-7 or KU-19-19, both of which are MAGE-3+ but HLA-A24− bladder cancer cells. On the other hand, CTL activity against FY cells was seen in the effector cells prepared from a nonrelated healthy donor with HLA-A24 typing, thus indicating that the basic mechanism underlying this modality requires matching for both HLA typing and tumor-specific peptide, which is expressed in target cells and is also presented to effector cells by DCs *in vitro*. These achievements using *in vitro* experiments formed the basis for conducting clinical trials with this DC-based immunotherapy in patients with advanced bladder cancers and histologically proven, measurable metastatic lesions. Our initial findings of clinical trials with only the four cases presented herein indicated that tumor-specific, DC-based cancer immunotherapy may be useful as a new additional treatment modality for advanced bladder cancer that is not curable by surgery, chemotherapy, or radiotherapy.

Numerous concerns still exist regarding the application of DCs in clinical treatment. Most of the patients with progressive cancers are likely to be of advanced age and to have a poor immunological status. The combination therapies with DCs and the systemic administration of cytokines are possible solutions. IL-12 is known to induce CTLs in patients with a poor immune

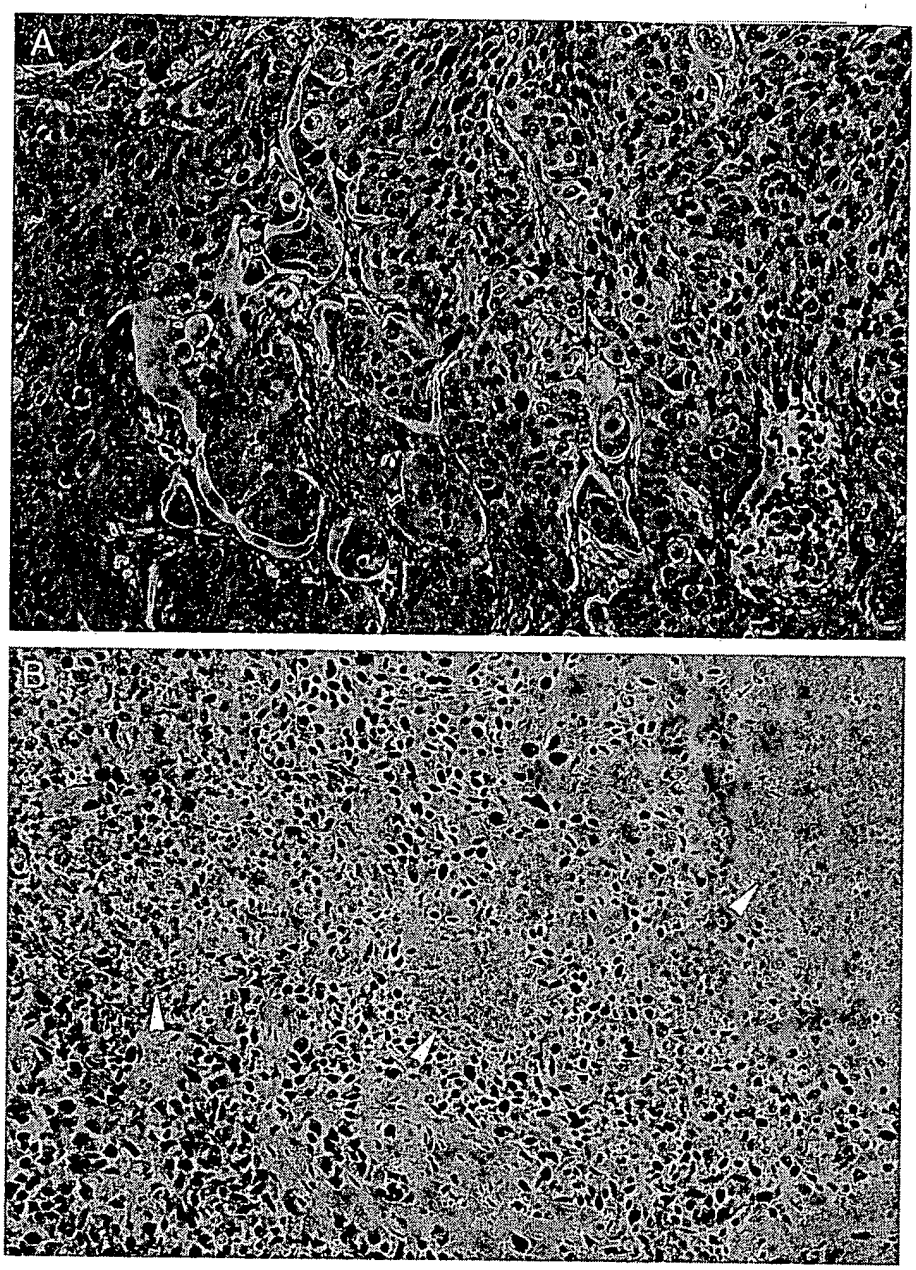

Fig. 5 Histopathological evaluation of representative biopsy specimens taken from inguinal lymph node metastasis of a bladder cancer patient before and after the MAGE peptide-pulsed DC immunotherapy. A typical transitional cell carcinoma was evident before the DC vaccination (*A*). Significant necrotic changes (*arrows*) were demonstrated 3 months after the DC vaccination (*B*; H&E, original magnification ×100).

status, and the concomitant use of IL-12 may reduce the need for large doses of DCs (43). In our preliminary experiments *in vitro*, when IL-12 was used to stimulate effector cells from PBMCs along with the autologous DCs pulsed with MAGE-3-encoded HLA-A24-binding epitope peptide, the CTL activity was significantly higher than the effector cells prepared without IL-12.[5] Additional studies based on these recent findings are now underway. Such studies should focus on the safety and the feasibility of using this DC-based active immunotherapy against advanced bladder cancers.

In summary, autologous DCs pulsed with HLA-A24-binding MAGE-3 epitope peptide have been shown to successfully induce MHC class I-restricted MAGE-3 specific autologous CTLs *in vitro* in MAGE-3+ bladder cancer. The efficacy of DCs pulsed with the epitope peptide remains to be elucidated, because its clinical application has just been initiated. However, our results clearly showed a good potential for the development

[5] T. Nishiyama, unpublished data.

of a DC-based, tumor-specific immunotherapy for the treatment of bladder cancer.

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